DEVELOPMENT OF A REAGENT-LESS SENSOR FOR INHIBITORS OF ACETYLCHOLINESTERASE

By

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SENSOR FOR INHIBITORS OF

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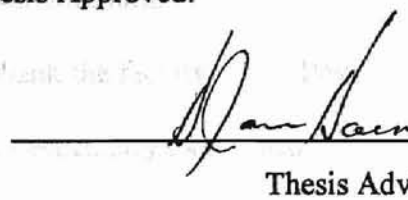
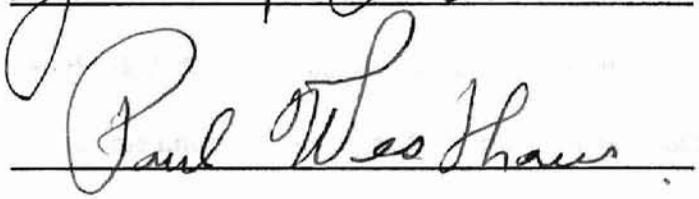
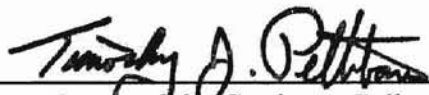
Thesis Approved:



Thesis Advisor





Dean of the Graduate College

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I would like to thank my advisor, Dr. H. James Harmon, for being there to support and guide me, for providing an excellent work environment, for tolerating my mood swings, and, most of all, for allowing me the freedom to learn. I look forward to continuing my work with him.

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NOMENCLATURE

Name	Abbreviation
Acetylcholinesterase	AChE
Acetylcholine Iodide	AChI
Acetylthiocholine	ATC
Dithiobisnitrobenzoic acid	DTNB
Monosulfonate tetraphenyl porphine	$TPPS_1$
Milliliter	mL
Millimolar	mM
Micromolar	μM
Nanomolar	nM
Nanometer	nm
Michaelis constant	K_m
Angstrom	Å

CHAPTER 1

1.1 PORPHYRINS

1.1.1 WHAT ARE PORPHYRINS

Porphyrins are nitrogen-containing compounds derived from the parent molecule tetrapyrroleporphin. Both natural and synthetic porphyrins exist. Some natural porphyrins are found in hemoglobin, myoglobin, cytochromes, and chlorophyll. Porphyrins are classified on the basis of the nature of the side chains replacing the hydrogens at positions 1-8 (Figure 1.1); methyl, ethyl, vinyl, and propionic acid are common substituents.

Formation of porphyrin-metal complexes results in altered porphyrin absorbance spectra. The spectral characteristics are dependent on both the metal involved and the solvent which is being used. This dependence on metal type is specific enough to allow differentiation between metals in a given solvent. The absorbance spectrum and the extinction coefficient of porphyrins and metalloporphyrins are known to be affected by the solvent. Solvent induced spectral changes result from the interactions effecting the pi-electron orbitals of the porphyrin. The same factors are involved in the spectral changes observed upon side chain substitution. Increases in pi-electrons cause red shifts of the absorbance and fluorescence bands while decreases in pi-electrons cause blue shifts (shorter wavelengths) of the absorbance and fluorescence bands (*1*).

Interaction with individual organic molecules strongly affects the spectral characteristics of porphyrins, as well (*2-4*). Amino acids (*5, 6*) and small peptides (*7, 8*) alter the absorbance spectra of porphyrins, each molecule resulting in unique spectral

characteristics (*3*). Porphyrin-protein interactions affect the spectral characteristics, as well, as shown in the interaction with serum albumin (*9-11*) and other proteins (*12*).

Porphyrins are known enzyme inhibitors that have been shown to inhibit AChE (*13, 14*). They have been studied in this capacity as photoactivated insecticides (*15*), and for use in treating Alzheimer's disease and *myasthenia gravis* (*16, 17*). Porphyrins have also been shown to inhibit telomerase and Hepatitis C virus serine protease (*18*). Metalloporphyrins have been shown to be potent inhibitors of human immunodeficiency virus (HIV) type 1 and 2 reverse transcriptases (*19*). Porphyrins have also been shown to bind to DNA (*20, 21*), and are used in photodynamic therapy (*22*).

1.1.2 PORPHYRINS AS SENSORS

The spectrophotometric characteristics of porphyrins allow for their application in a wide variety of situations as colorimetric indicators. A large volume of literature reflects the interest in porphyrins for use in molecular recognition. Porphyrins have been used for detection of sugars, alcohols, amino acids, amines, DNA, and quinones in solution. The uniqueness of the interactions, providing specific spectrophotometric markers in the UV/vis and fluorescence spectra for different compounds, allows for wide application of porphyrins in this field. In the case of amino acids, interaction specificity allows for determination of chirality based on the peak positions resulting from the interaction (*23*). Reflectivity measurements have been similarly used. A surface coated with gold has been used as a foundation for a film of porphyrins. The spectrum of reflected light or the intensity of a particular wavelength is then affected by exposure to interactive compounds (*24, 25*).

Porphyrinic sensors for detection of electroactive analytes can also make use of the formation of thin films of metalloporphyrins on the surface of an electrode. In this way it is possible to detect the oxidation or reduction of the analyte based on the current generated. Specificity arises from the possibility of modifying the side chain constituents and the incorporated metal of the porphyrin, therefore altering its redox properties. This type of sensor is used for detection of metal cations and nitric oxide (*26, 27*).

Anion-selective potentiometric sensors can be based on the doping of non-conductive membranes with metalloporphyrins. This incorporation requires interaction of the anion as a ligand. The ability of the anion to interact with the porphyrin is therefore restricted by the metal incorporated and the side chains involved. This type of sensor can be used for detection of compounds such as salicylate, nitrite, and 2-hydroxybenzyhydroxamate (a therapeutic agent) as well as nickel (*27-29*).

1.2 ACETYLCHOLINESTERASE

1.2.1 STRUCTURE AND FUNCTION

Acetylcholinesterase (AChE) is a 537 amino acid residue protein containing 12 strand β-sheets and 14 α-helices. The x-ray crystallographic structure has been determined to 2.8 Å resolution (*30*). The primary biological role of AChE is the termination of impulse transmission at the cholinergic synapses. This is accomplished by hydrolyzing the neurotransmitter acetylcholine (ACh) to acetate and choline.

The active site of AChE lies in a deep narrow gorge, which penetrates nearly to the center of the protein. The active site is composed of two subsites. The esteratic site resembles that of the other serine hydrolases and requires major participation of the Ser^{200}

residue. Also involved in binding ACh, are Trp^{84}, Gly^{118}, Gly^{119}, and Ala^{201}. The ACh is guided into the active site by the many aromatic residues, which line the active site gorge.

Inhibitors of AChE are those compounds which block the hydrolysis of the substrate acetylcholine, resulting in slower catalytic rates and therefore larger Michaelis constants (K_m). Inhibitors may bind in the active site or at a peripheral site. Those chemicals, which bind at the active site, are called competitive inhibitors. They do not effect the maximal catalytic rate, V_{max}. Chemicals binding to the peripheral sites and resulting in larger K_m values are mixed inhibitors. These inhibitors will cause a change in the V_{max} value.

The importance of AChE is its basis as a target for attack. The inhibition of AChE is used as a method of pest control and as a weapon. Because organophosphate compounds, which are the major constituents of many insecticides and nerve agents, covalently bind to the serine residue, they semi-permanently or permanently, depending on the compound, block the hydrolysis of ACh. When this happens, the nerve impulse receptors constantly receive signals. In humans, this overstimulation causes salivation, sweating, loss of muscle control, spasm, and death. Death is normally the result of respiratory failure (*32*). More recently, reversible inhibition of AChE has been tested as a method of treating diseases such as Alzheimer's (*16*).

1.2.2 USE AS A SENSOR

Since the catalytic activity of a given amount of AChE is reduced upon exposure to an organophosphate, a measurement in the change in activity can be used to detect the probable presence of one of these chemicals. There are several sensors based on this idea.

Bachmann and co-workers describe a method of monitoring immobilized AChE activity by electrochemically monitoring oxidation of thiocholine produced upon hydrolysis of ATC. A baseline of AChE activity must first be collected before a sample can be exposed to inhibitors. After incubation with the preferred inhibitor, the AChE activity is again measured. The before and after activity are compared to determine the level of inhibition of the enzyme and therefore the concentration of the inhibitor. This method is also used for detection of cholinesterase-inhibiting insecticides (*33*).

This is not a unique method of detection for enzyme inhibitors. It is employed repeatedly using different techniques for monitoring activity of AChE, but in all cases a baseline of catalytic activity is required. This method is not limited to application with AChE. It is also used with other enzymes, such as choline oxidase, butyrylcholinesterase, organophosphorus hydrolase (*33-59*). It would be better if a method of determination could be developed which did not require a baseline of enzyme activity. Enzyme stability increases upon immobilization, however, activity will decrease as the enzyme ages. In addition, sensitivity may be limited by the method of determination of enzymatic activity. A low concentration of inhibitor may not be sufficient to alter the enzymatic rate noticeably. These factors discourage the use of a sensor of this type other than in a lab environment.

The need for a detector of chemical warfare agents for use by first-responders and military personnel is intense and ongoing. An ideal sensor would be one that is agent-specific with a minimum of interferents and false positive readings and that is compact, battery-powered, has a long shelf life, is sensitive, and reports in real-time. Devices with as many of these characteristics as possible are still sought.

Current sensors of nerve agents that inhibit acetylcholinesterasè are based primarily upon comparison of catalytic rates of acetylcholine esterase at a given time to a baseline, background, or pre-exposure level (*34-58, 60, 61*). The measurement of catalytic rates may involve multiple steps involving the addition or changing of one or more reagent solutions, a somewhat undesirable operating characteristic.

Sensors can be broadly classified into two groups: detectors of specific agents (e.g., VX) (*6, 40-42, 49, 62*) and detectors of AChE inhibitors in general (*12, 34, 38, 45, 46, 48, 51, 53-55, 57, 58*). There is a need for both types of sensor. The central core technology of any sensor/detector is a system that it transduces the presence of the chemical into an optical, electronic, or other signal that can be processed either by eye or electronic circuitry.

THE PORPHYRIN MOLECULE

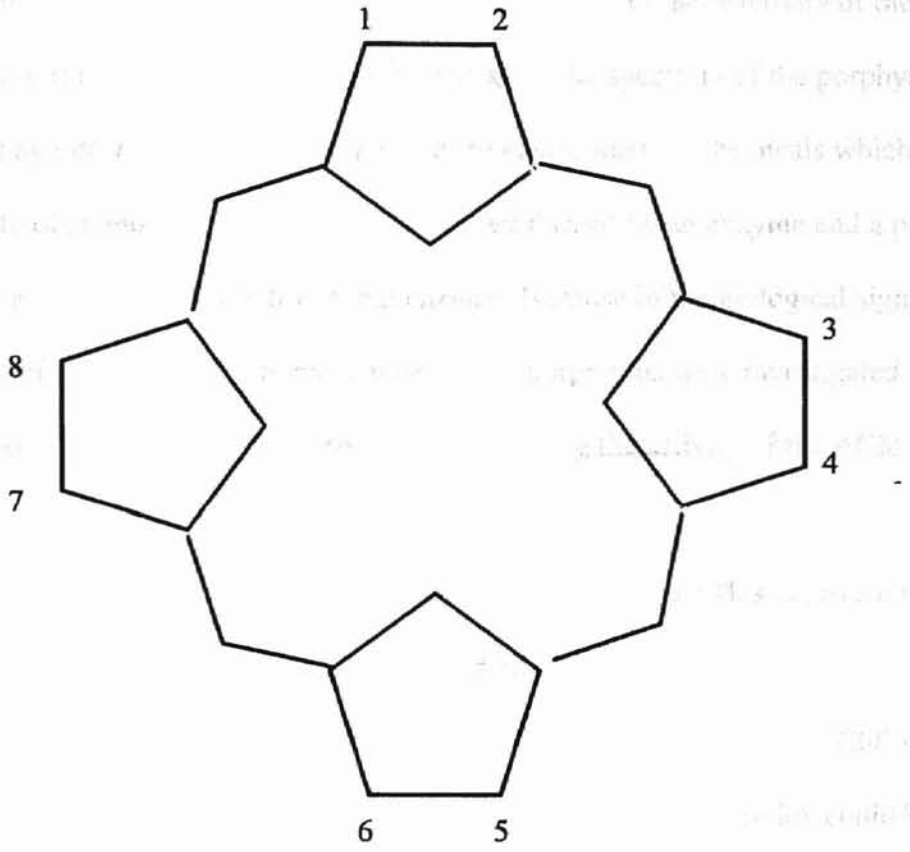

FIGURE 1.1 The structure of the parent molecule, tetrapyrroleporphin, from which porphyrins are derived. Hydrogens at positions 1-8 are replaced by the side chains for the porphyrins.

CHAPTER 2

2.1 THE RESEACH IDEA

Porphyrin interactions with proteins have been widely studied and are being applied in many ways, as mentioned in Chapter 1. Of especial interest are the changes in the spectrum of porphyrins upon binding to an enzyme and the fact that some porphyrins have been shown to be competitive inhibitors (*14*). Due to the reactivity of the porphyrins, it may be possible to cause a change in the spectrum of the porphyrin-enzyme complex by introducing other chemicals to the environment. Chemicals which bind the active site of an enzyme may effect the complex formed by an enzyme and a porphyrin which is a competitive inhibitor of that enzyme. Because of the biological significance of acetylcholinesterase, interactions between it and porphyrins were investigated.

AChE has been used as a sensor by measuring the activity of the AChE remaining uninhibited after introduction of an inhibitor to the environment. This indicates the percentage of the AChE which has presumably been affected. This approach requires a baseline of AChE activity for comparison and allows for introduction of error due to enzyme instability. The preferable approach would be to measure the AChE which had been affected by the inhibitor directly. If a porphyrin-enzyme complex could be formed with spectral characteristics which are sensitive to inhibitors of AChE, a colorimetric sensor could be based on those changes.

In the pursuit of this idea, porphyrins were tested for their ability to inhibit, and therefore bind, AChE. Such a porphyrin was then investigated for the type of interaction it displayed with AChE. Questions addressed were the following:

1. Do the porphyrin and enzyme interact?

2. Does this interaction yield a spectral signature?
3. What is the nature of the interaction?
4. What, if any, are the effects on the spectral signature upon addition of substrate to the environment?
5. What, if any, are the effects on the spectral signature upon addition of other known AChE inhibitors?
6. What are the limits of sensitivity for the changes caused by competitive inhibitors?

2.2 MATERIALS AND METHODS

Acetylcholinesterase (AChE, type V-S from electric eels), tetracaine, procaine HCl, acetylcholine iodide (AChI), dithiobisnitrobenzoic acid (DTNB) and acetylthiocholine iodide (ATC) were obtained from Sigma (St. Louis, MO) and used without further purification. Monosulfonate tetraphenyl porphine ($TPPS_1$, Figure 2.1) was obtained from Frontier Scientific (Logan, UT).

Enzyme assays were conducted in 3 mL of 100 mM pH 8 phosphate buffer maintained at 25C according to the method of Ellman (*63*) with slight modifications: (1) reaction rates were measured using a Gilford single beam spectrophotometer and (2) AChE was dissolved in 100 mM pH 7 phosphate buffer instead of gelatin and water. In order to avoid possible contributions from the absorption of $TPPS_1$ (λ_{max} = 405 nm), reaction rates were measured at 435 nm instead of 412 nm. The absorbance of the yellow anion of 5-thio-2-nitro-benzoic acid, the result of the DTNB-thiol reaction, at 435 nm is 76% of that at 412 nm.

Absorbance spectra of $TPPS_1$ and $TPPS_1$ in the presence of AChE, tetracaine, procaine, and AChI were recorded in 5 mM pH 8 sodium phosphate (Sorenson) buffer

with a Cary 4E spectrophotometer at 0.02 nm resolution. Difference spectra were obtained by subtraction of absolute spectra using Grams/32 (Galactic Industries, Salem, NH). Fluorescence spectra of $TPPS_1$ in the presence/absence of AChE were recorded in 5 mM pH 8 sodium phosphate (Sorenson) buffer. Fluorescence spectra were collected with a Spex Fluromax 3 spectrofluorometer using 2.5 nm resolution for excitation wavelength and 1.0 nm resolution for emission wavelength. Final reagent concentrations are given in the figure captions. Spectral analysis was again preformed using Grams/32 software.

2.3 RESULTS AND DISSCUSION

2.3.1 PORPHYRIN-ENZYME INTERACTION

The first points to be addressed are the presence/absence of interaction between $TPPS_1$ and AChE and, if applicable, the type of interaction between them. The absorbance spectrum of monosulfonate tetraphenyl porphine ($TPPS_1$) is shown in Figure 2.2 (Trace 1). $TPPS_1$ has absorbance bands in addition to those shown here at 518, 552, 591, and 647 nm. For this research the focus will be in the 350 to 500 nm region where the Soret is located. The peak shown in this figure is composed of three bands, likely due to different species of $TPPS_1$. Upon binding of AChE, a peak shift to longer wavelengths occurs as shown in Figure 2.2 (Trace 2), which is more clearly observed in the $TPPS_1$ + AChE minus $TPPS_1$ difference spectrum (Figure 2.2, Trace 3). The difference spectrum, which shows the changes in the $TPPS_1$ absorbance due to enzyme binding, displays a new absorption peak at 442 nm resulting from the $TPPS_1$-AChE complex and a trough at

402 nm resulting from a decrease in the amount of unbound $TPPS_1$. This spectral shift is independent of pH (7 to 11) and independent of salt concentration (2 mM to 100 mM).

The intensity of the peak at 442 nm resulting from the $TPPS_1$-AChE interaction is dependent on AChE concentration as shown in Figure 2.3. The inflection point occurs at 120 nM AChE. On the basis of the extinction coefficient (E = 500 cm/mM), the absorbance increase at 442 nm corresponds to 110 nM $TPPS_1$. Therefore, there is approximately one $TPPS_1$ per AChE. The dependence is linear up to 140 nM AChE, after which point the absorbance does not increase.

The fluorescence spectra resulting from excitation of $TPPS_1$ alone from 375 nm to 450 nm with emission measured from 600 nm to 750 nm are shown in Figure 2.4. The peak fluorescence intensity occurs at 652 nm achieving a maximum when excited at 406 nm. In addition, a fluorescence band is observed in the region between 690 and 740 nm with maximum intensity achieved also at 406 nm excitation. The peak emission intensity of this region is at 712 nm. These bands are more prominent when viewed as a contour plot (Figure 2.4).

Acetylcholinesterase alone does not exhibit fluorescence when excited from 375 nm to 450 nm (data not shown). Excitation of $TPPS_1$ bound to AChE, however, results in a narrowing of the fluorescence band at 652 nm (Figure 2.5). The $TPPS_1$-enzyme complex also shows maximum emission and excitation wavelengths in the fluorescence bands in the region from 690 nm to 740 nm. Figure 2.6 shows the fluorescence intensity for $TPPS_1$ alone (Figure 2.6, Panel A) and in the presence of AChE (Figure 2.6, Panel B) at 652 nm emission for all excitation wavelengths. The addition of AChE does not affect the emission peak for this band. A shift is observed in the maximum intensity excitation

wavelength from 407 nm to 412 nm (Figure 2.6). The band in the region between 690 nm and 740 nm displays shifts in both peak emission and excitation wavelengths. The peak intensity of these bands is shifted from 712 nm to 716 nm (Figure 2.5) and the excitation wavelength at which maximum intensity occurs shifts from 406 nm to 413 nm (Figure 2.7, Panels A and B).

It is obvious from the fluorescence and absorbance data that $TPPS_1$ and AChE interact. Formation of a $TPPS_1$-AChE complex yields a very specific change in the absorbance spectrum and several changes in the fluorescence spectrum of $TPPS_1$.

The next point to be addressed is the type of interaction which exits between $TPPS_1$ and AChE. The Lineweaver-Burk plot of AChE activity at different substrate concentrations in the absence and presence of 470 nM $TPPS_1$ is shown in Figure 2.8. The linear plots intersect on the Y-axis; the K_m values for ATC are 87 and 141 μM in the absence and presence of $TPPS_1$ respectively. The intersection of the lines on the Y-axis indicates that $TPPS_1$ is a competitive inhibitor of AChE. This finding is consistent with the findings of Lee and co-workers (*13, 14*), who suggest that porphyrins show either mixed or competitive inhibition. Our data supports the latter possibility. In order to competitively inhibit an enzyme, the compound in question must bind in the active site (*64*). So from this data, we can determine that not only do $TPPS_1$ and AChE interact but $TPPS_1$ binds to the active site of the enzyme.

2.3.2 EFFECT OF SUBSTRATE ADDITION $TPPS_1$-ENZYME COMPLEX

The difference spectrum $TPPS_1$ + AChE + AChI <u>minus</u> $TPPS_1$, resulting from addition of AChI and AChE to $TPPS_1$, shows the combined effect of substrate and enzyme on $TPPS_1$ (Figure 2.9, Trace 1). The difference spectrum displays a peak at 447

nm and trough at 402 nm. The $TPPS_1$ + AChE + AChI minus $TPPS_1$ + AChE difference spectrum (Figure 2.9, Trace 2), which shows the change in the $TPPS_1$ bound to AChE due to the addition of the substrate, displays a narrowed peak at 447 nm and a trough at 402 nm. The lack of an absorbance peak at 442 nm [shoulder on 447 nm peak (cf. Figure 2.9, Trace 2); its non-presence results in the "narrowing" of the 447 nm peak] in the presence of substrate compared to the absence of substrate as seen in Figure 2.9 (Trace 3) indicates the dissociation of $TPPS_1$ from the enzyme due to the competitive binding of the substrate ($TPPS_1$ in solution does not show a 442 nm peak; 442 nm peak is only seen when $TPPS_1$ is bound to AChE).

When we subtract Trace 2 of Figure 2.9 from Trace 1 of Figure 2.9, the result (Figure 2.9, Trace 3) shows the same spectral characteristics as $TPPS_1$ + AChE minus $TPPS_1$ (Figure 2.2, Trace 3). Since Figure 2.9 Trace 1 is the effect of substrate and enzyme on $TPPS_1$ and Figure 2.9 Trace 2 is the change in the $TPPS_1$ by the enzyme, the difference in the $TPPS_1$ is likely attributable to the dissociation of the $TPPS_1$-enzyme complex.

That this is so is also shown by the difference spectrum of $TPPS_1$ + AChI minus $TPPS_1$ (indicates the effect of substrate on the $TPPS_1$ spectrum) which shows a peak at 447 nm (Table 2.1). Thus, the presence of the 447 nm peak and 442 nm trough (seen as a shoulder on the 402 nm trough) in the $TPPS_1$ + AChE + AChI minus $TPPS_1$ + AChE difference spectrum are due to the changes in the $TPPS_1$ spectrum resulting from formation of $TPPS_1$-AChI complex as well as dissociation of the $TPPS_1$-enzyme complex. The narrowed 447 nm peak in the $TPPS_1$ + AChE + AChI minus $TPPS_1$ + AChE difference spectrum is due to the loss of the 442 nm band. As AChI displaces

$TPPS_1$ from the active site, the 442 nm band due to $TPPS_1$ binding AChE becomes smaller.

2.3.3 EFFECT OF ACHE INHIBITORS

Tetracaine has been shown to be a competitive inhibitor of AChE (*65, 66*) while procaine has not, suggesting that tetracaine binds to the active site while procaine binds elsewhere, likely at the peripheral site located adjacent to the active site (*65*). In the presence of tetracaine, the $TPPS_1$ + AChE + tetracaine minus $TPPS_1$ difference spectrum (Figure 2.10, Trace 1) displays a trough at 402 nm and peak at 445 nm. The loss of the porphyrin-enzyme complex, represented by an absorbance band at 442 nm, is consistent with tetracaine displacing the $TPPS_1$ from the enzyme active site.

By comparison, the loss of the 442 nm peak is not observed when procaine (2.0 mM) is used in place of tetracaine (Figure 2.10, Trace2). The $TPPS_1$ + AChE + procaine minus $TPPS_1$ difference spectrum shows peaks at 442 nm and 429 nm and a trough at 402 nm. The interaction between $TPPS_1$ and AChE is still present (442 nm band) and we observe an interaction between $TPPS_1$ and the procaine represented by the absorbance peak at 429 nm (Table 2.1). The binding of an inhibitor at a site other than the active site, therefore, does not effect the $TPPS_1$-enzyme interaction strongly enough to change the 442 nm absorbance characteristic.

2.4 CONCLUSIONS

Based on the presented data, $TPPS_1$ binds to the active site of AChE in a one to one ratio. The formation of the $TPPS_1$-AChE complex results in a change in the spectral characteristics of $TPPS_1$. The enzyme-porphyrin complex then exhibits spectral

characteristics sensitive to the presence of chemicals which bind to the active site of AChE.

It is interesting to note that it may be possible to use porphyrins to detect the presence of specific proteins. Shifts in absorbance and fluorescence spectra tend to be unique for different chemicals (*3*) including different proteins (*9*). Spectral shifts could be used as a "marker" for the presence of proteinaceous bacterial exotoxins. This category includes botulinum toxin and those found in *Clostridium botulinum*, *Clostridium tetani*, *Clostridium perfringens*, *Escherichia coli*, cholera, and plague (*Yersinia pestis*).

THE $TPPS_1$ MOLECULE

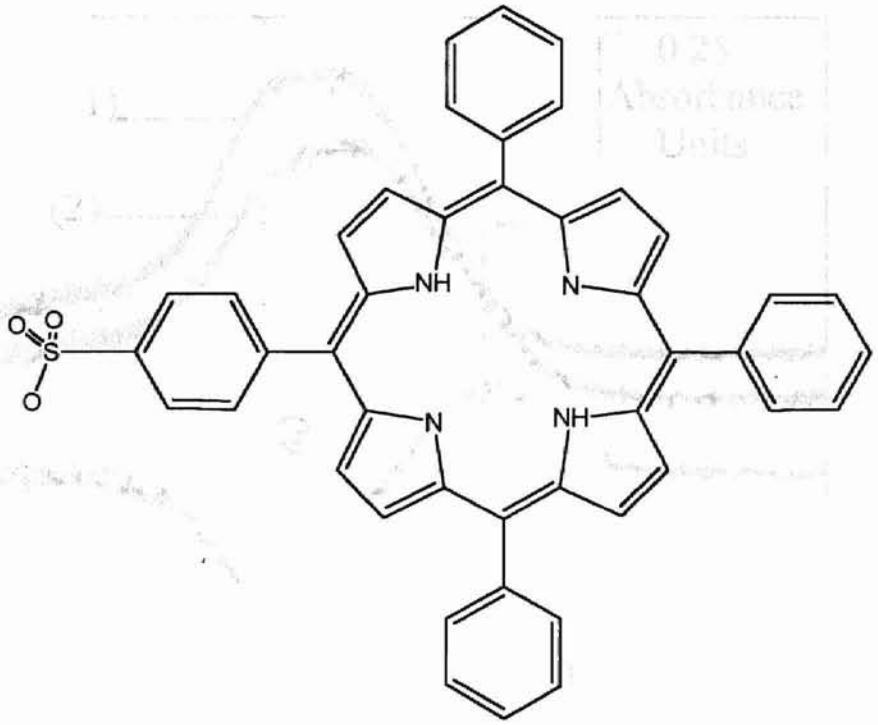

FIGURE 2.1 Monosulfonate tetraphenyl porphine has three hydrophobic phenyl groups and one sulfonate group on its periphery.

EFFECT OF ACHE ON THE $TPPS_1$ SPECTRUM

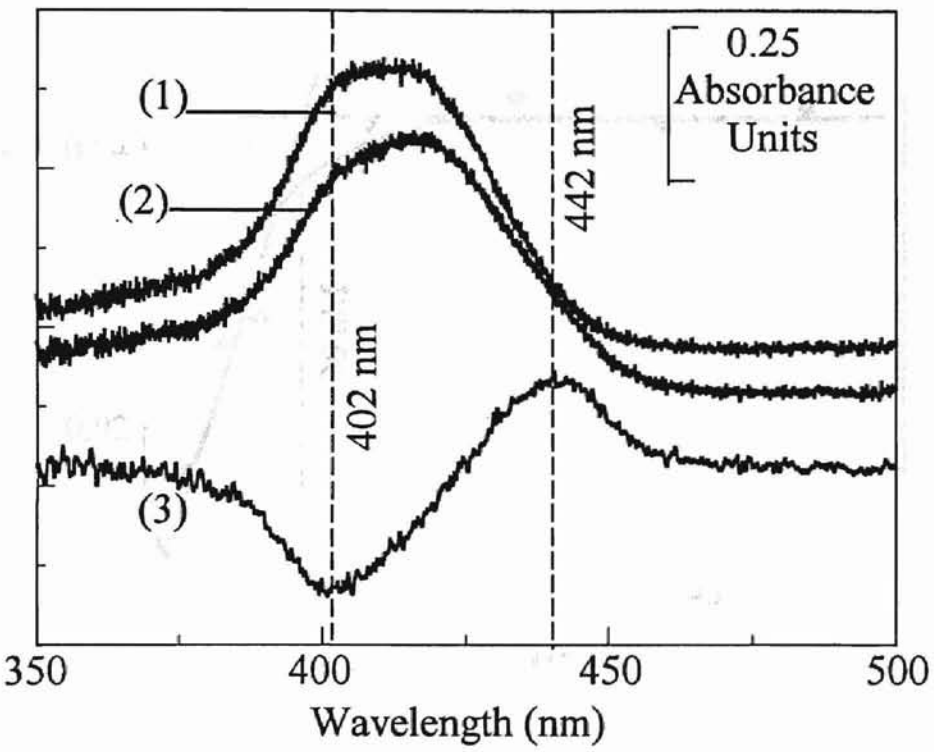

FIGURE 2.2 The absolute spectrum of 730 nM $TPPS_1$ (Trace 1) and the absolute spectrum of 730 nM $TPPS_1$ + 30 nM AChE (Trace 2) show the shift, which occurs upon formation of the $TPPS_1$-AChE complex. The difference spectrum $TPPS_1$ + AChE – $TPPS_1$ (Trace 3) shows interaction peaks.

DEPENDENCE OF INTERACTION ON ACHE CONCENTRATION

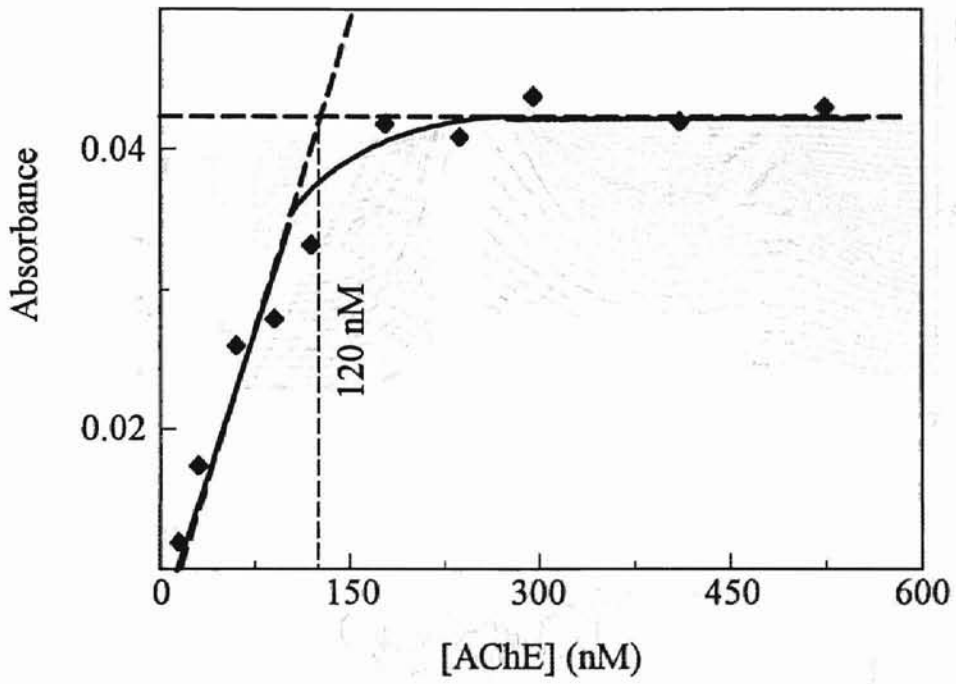

FIGURE 2.3 The dependence on AChE concentration of the peak at 442 nm in the difference spectrum $TPPS_1$ + AChE minus $TPPS_1$.

FLUORESCENCE SPECTRUM OF $TPPS_1$

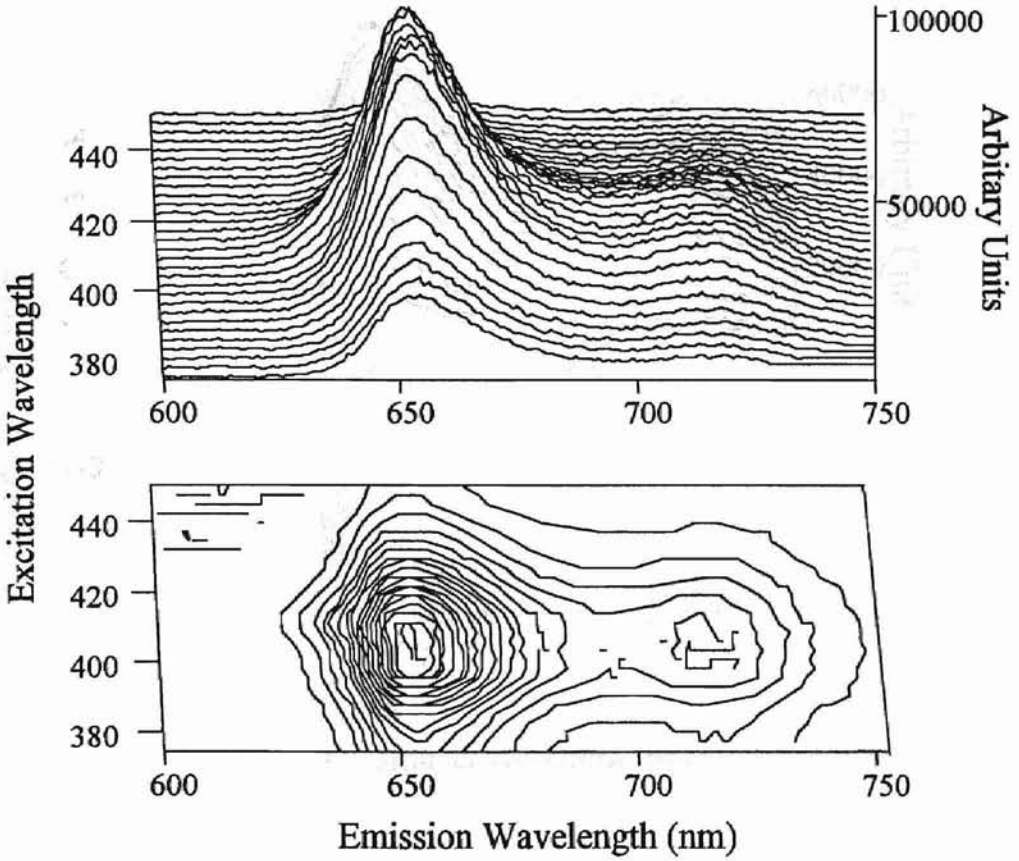

FIGURE 2.4 The fluorescence of $TPPS_1$ shows two major band groups. The strongest has a peak of emission at 652 nm while the other shows a peak of emission at 712 nm.

FLUORESCENCE SPECTRUM OF $TPPS_1$ IN THE PRESENCE OF ACHE

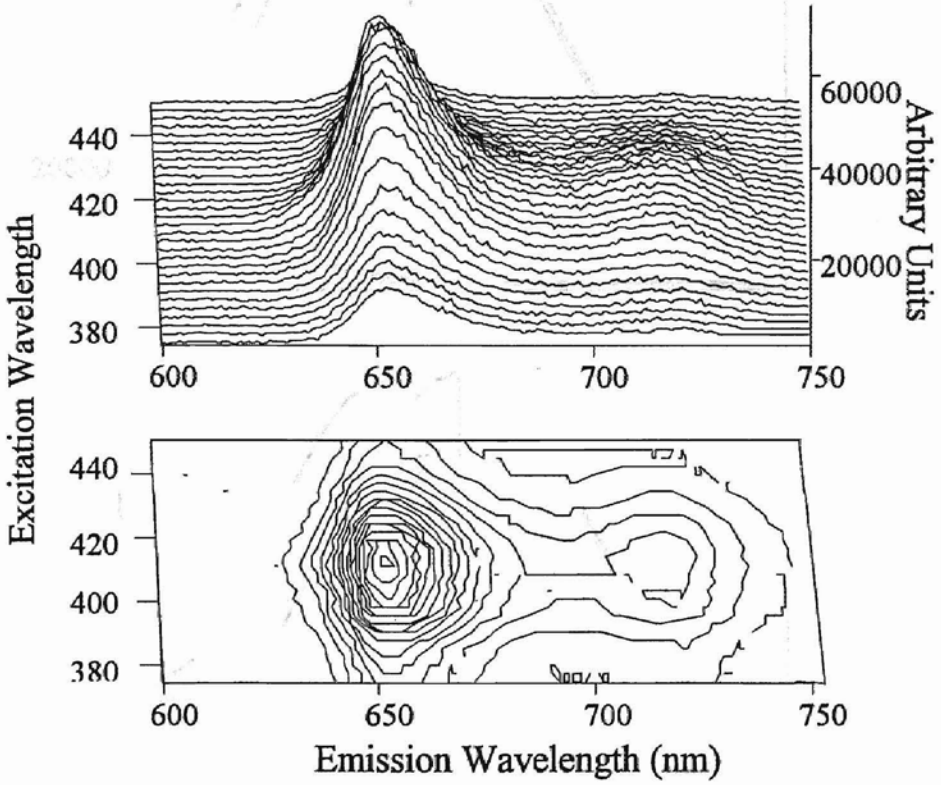

FIGURE 2.5 The fluorescence of $TPPS_1$ in the presence of AChE shows two major band groups. The strongest emission band has a peak at 652 nm while the other shows a peak at 716 nm.

EMISSION INTENSITY OF $TPPS_1$ AT 652 NM IN THE ABSENCE / PRESENCE OF ACHE

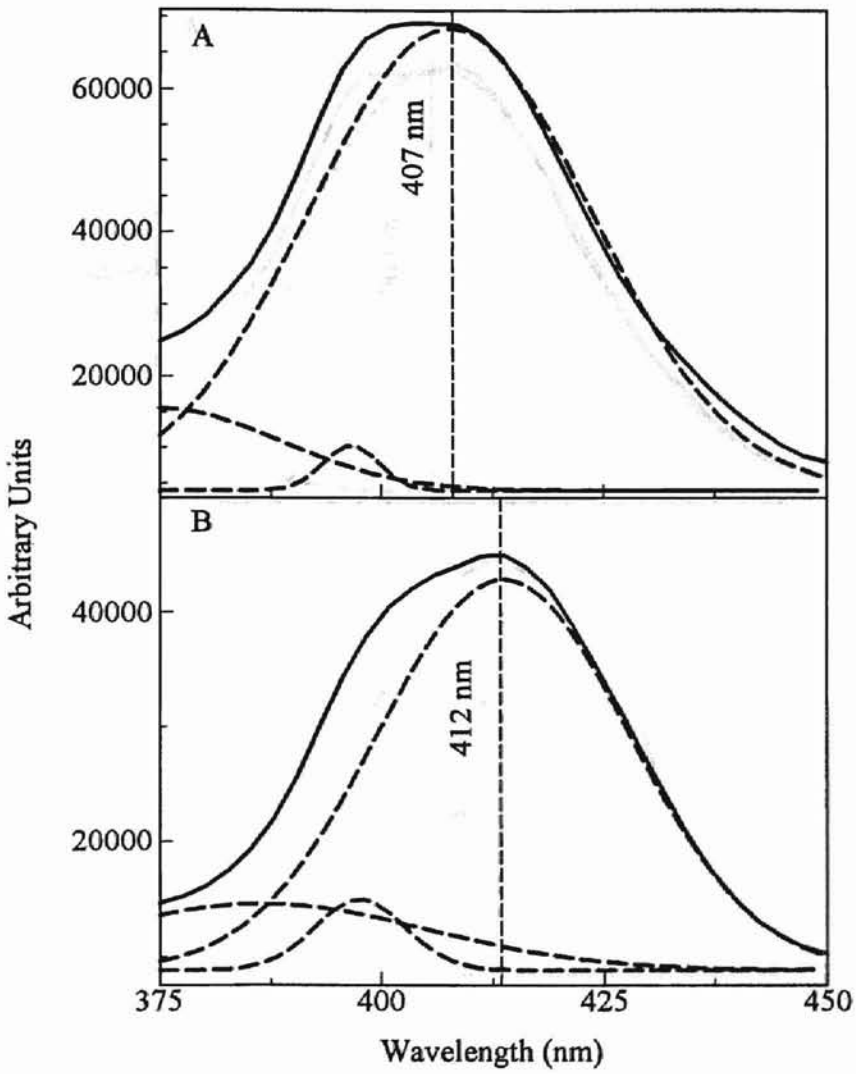

FIGURE 2.6 The fluorescence of $TPPS_1$ in the presence (Panel A) / absence (Panel B) of AChE at 652 nm, the peak emission intensity. A shift in the peak excitation wavelength from 407 nm to 412 nm is observed upon addition of AChE.

EMISSION INTENSITY OF $TPPS_1$ AT 712 NM AND $TPPS_1$ IN THE PRESENCE OF ACHE AT 716 NM

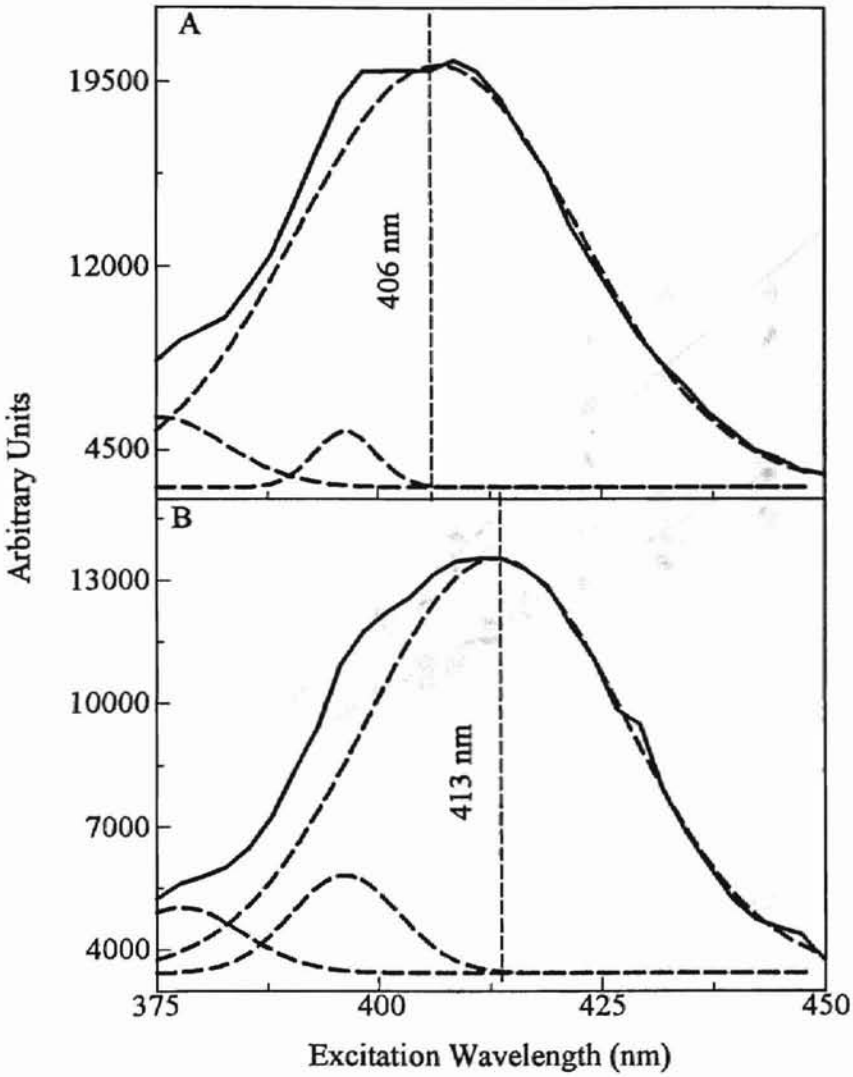

FIGURE 2.7 The fluorescence of $TPPS_1$ in the absence of AChE (Panel A) shows a peak at 712 nm with the peak excitation wavelength at 406 nm. In the presence of AChE (Panel B), the peak emission is shifted to 716 nm with peak excitation at 413 nm.

LINEWEAVER-BURK PLOT OF ACHE ACTIVITY

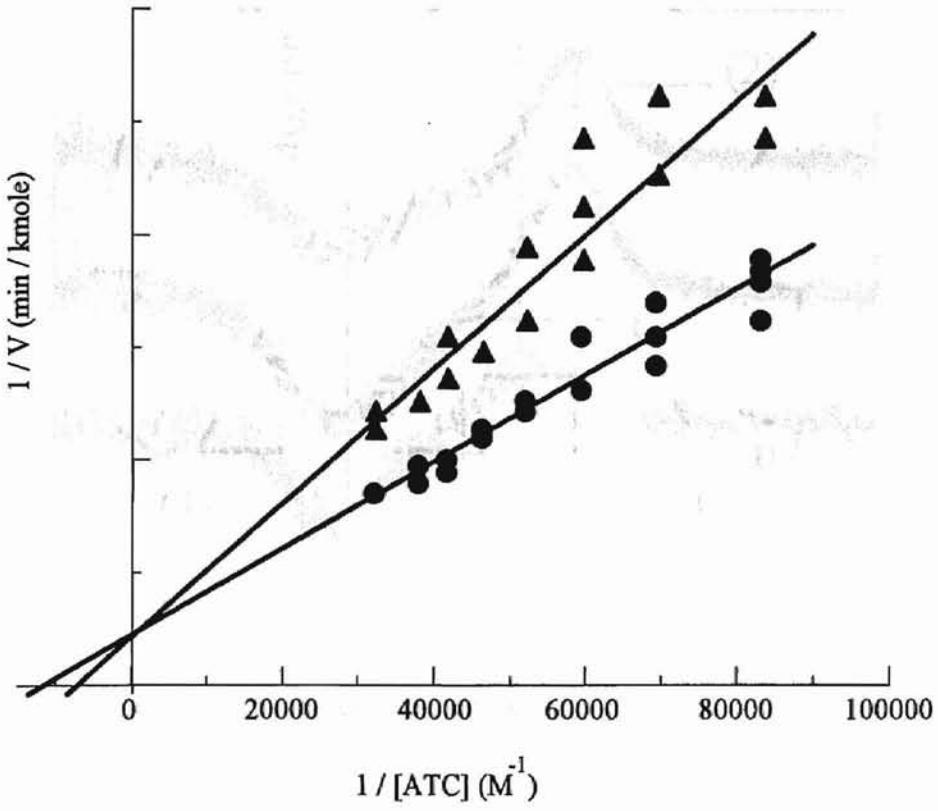

FIGURE 2.8 The Lineweaver-Burk plot of AChE activity at different ATC concentrations is shown as 1/V vs. 1/[S], where V is in kmole per minute. In the absence of inhibitor (●) K_m = 87 μM. In the presence of 470 nM $TPPS_1$ (▲) K_m = 141 μM. Intersection of the curves occurs at the Y-axis, V_{max} = 0.02 kmol/min.

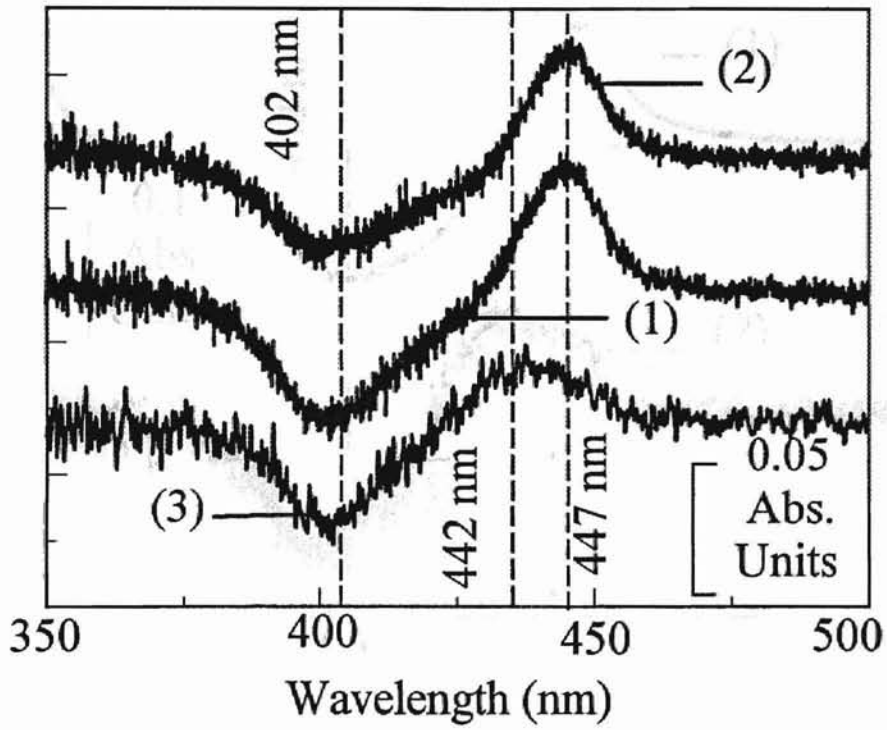

FIGURE 2.9 Spectral changes in the $TPPS_1$-AChE complex upon addition of AChI. Trace 1: the difference spectrum of $TPPS_1$ + AChE + AChI minus $TPPS_1$; Trace 2: the difference spectrum of $TPPS_1$ (730 nM) + AChE (30 nM) + AChI (18 μM) minus $TPPS_1$ + AChE, Trace 3: the double difference spectrum of ($TPPS_1$ + AChE + AChI minus $TPPS_1$) minus ($TPPS_1$ + AChE + AChI minus $TPPS_1$ + AChE) yields $TPPS_1$ + AChE minus $TPPS_1$.

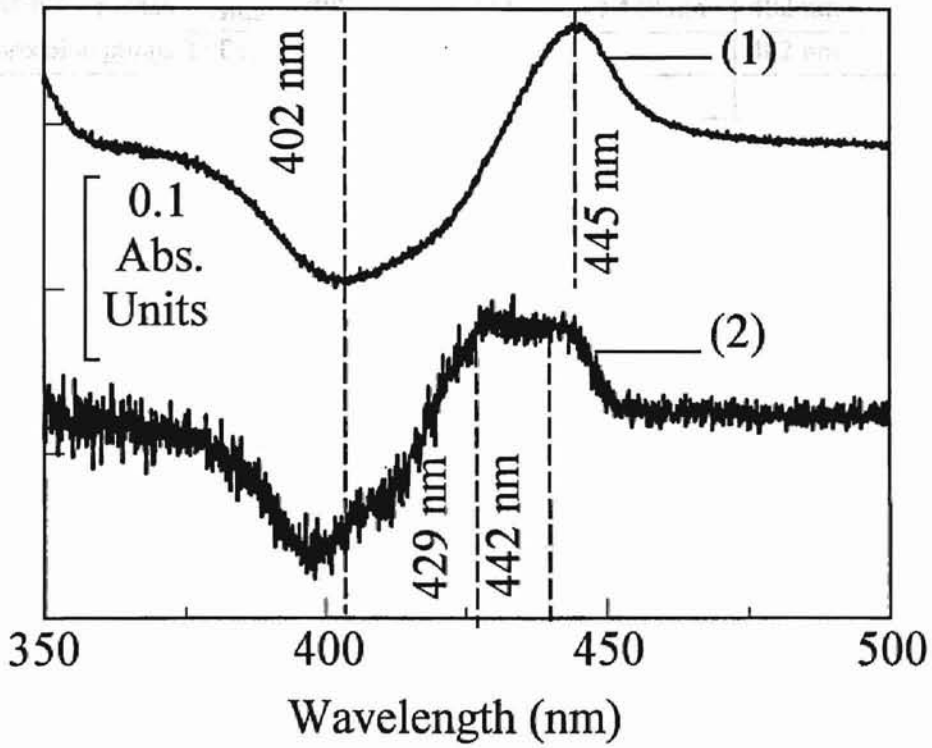

FIGURE 2.10 Trace 1: The interaction of the $TPPS_1$-AChE complex with tetracaine. The difference spectrum of $TPPS_1$ (730 nM) + AChE (30 nM) + tetracaine (0.5 mM) minus $TPPS_1$. Trace 2: The difference spectrum of $TPPS_1$ (730 nM) + AChE (30 nM) + procaine (2.0 mM) minus $TPPS_1$ + procaine.

DIFFERENCE SPECTRUM BAND POSITIONS

Difference Spectrum	Peak	Trough
$TPPS_1$ + AChE minus $TPPS_1$	442 nm	402 nm
$TPPS_1$ + AChE + AChI minus $TPPS_1$ + AChE	447 nm	402 nm and 442 nm
$TPPS_1$ + AChE + AChI minus $TPPS_1$	447nm	402 nm
$TPPS_1$ + AChI minus $TPPS_1$	447 nm	402 nm
$TPPS_1$ + AChE + tetracaine minus $TPPS_1$	445 nm	402 nm
$TPPS_1$ + tetracaine minus $TPPS_1$	445 nm	402nm
$TPPS_1$ + AChE + procaine minus $TPPS_1$	442 nm and 429 nm	402 nm
$TPPS_1$ + procaine minus $TPPS_1$	429 nm	402 nm

TABLE 2.1 The peak and trough positions in the difference spectra resulting from the interaction of $TPPS_1$ with the enzyme and each of the inhibitors.

CHAPTER 3

Use of the AChE-$TPPS_1$ complex as a sensor to be applied outside of a lab environment necessitates the formation of a system more durable and versatile than that of a solution. Mixing of reagents in the field is not practical due to the time involved, necessity of exact measurements, and storage requirements. AChE has been proven stable for a few weeks only if stored frozen. Enzymes are typically more stable when they are immobilized. In the interest of convenience and application in a variety of situations, the formation of a layer of immobilized AChE has been investigated.

Several procedures for immobilizing enzymes are sited in the literature. Acetylcholinesterase contains a total of 76 amino group bearing residues; 26 lysine, 15 glutamine, 35 asparagine. These amino residues can be used to immobilize by binding to a Schiff base formed from another amino group. A brief review of a few methods follows, as well as, a summary of the progression of the immobilization technique to be employed.

3.1 STANDARD IMMOBILIZATION METHODS

3.1.1 IMMOBILIZATION ONTO NYLON NETS

One commonly used method of immobilization is accomplished through the use of nylon net. This procedure is described by Mascini and co-workers (*67*). The nylon netting that is used consists of 120 threads/cm each having a thickness of 100 μM. The nets are immersed in dimethyl sulfate at 100 C for five minutes followed by immersion in an ice bath to stop the reaction. The nets are then immersed in anhydrous methanol for several minutes followed immediately by immersion in lysine solution to coat the surface with amine-containing lysine. The nets are treated with glutaraldehyde to encourage

formation of Schiff bases at the free amino groups. Finally the nets are immersed in the enzyme solution. This type of immobilized enzyme has been used to make hydrogen peroxide electrodes, oxygen electrodes, and Platinum electrodes (*51, 54, 67*).

3.1.2 IMMOBILIZAION ONTO GLASS

A method for immobilization of AChE onto glass has been described by Leon-Gonzales and Townshend (*57*). The glass described is controlled porosity glass, CPG-240, obtained from Sigma Chemical. An aminoalkylating agent, 3-aminopropyltriethoxysilane (3-APTES), is reacted with the glass. After alkylamination, the alkylamino glass is activated with glutaraldehyde. After being thoroughly rinsed, the activated glass is reacted with AChE. This method has been used for flow injection measurements of enzyme inhibition (*53, 57*).

A slightly modified procedure is used for the treatment of silica gel by Simonian and co-workers (*49*). They use an acetone wash following the treatment with 3-APTES. Activation of amino groups is also achieved through the use of glutaraldehyde. The enzyme treated gels have been used for flow injection experiments as well as with pH electrodes (*49, 52*).

3.2 DEVELOPMENT OF DENDRIMER TECHNIQUE

3.2.1 THE EARLY TESTS

Xenobind microwell plates (Xenopore, Saddle Brook, NJ) were originally used for binding of AChE. These plates are designed to form covalent bonds with the amino groups on the surface of proteins. The binding procedure recommended by Xenopore was followed. The protein was dissolved in buffer and allowed to interact with the surface for several hours. The excess protein was then washed away and the remaining

sites blocked with casein. The result was a surface with density quite low for the purposes we were trying to achieve. This type of well also had a short lifetime based on the decrease and cessation of catalytic activity over a period of a few days. Enzyme activity was measured using the method of Ellman and co-workers discussed in Part III.

A surface with higher enzyme density was required, so DNA-Bind microwell plates (Coastar, Cambridge, MA) were investigated. DNA-Bind surfaces are again designed to interact with protein surface amino groups. Binding of AChE to the DNA-Bind plates was attempted using the method described for the Xenobind plates. With much the same results achieved. The DNA-Bind wells displayed low enzyme density and lifetimes of only a few days. A modification of the procedure used has proven much more successful.

For the new procedure, activation of the wells with glutaraldehyde is followed by interaction with Starburst. Starburst is a generation 4 PAMAM dendrimer from Aldrich Chemical Co. (Milwaukee, WI). This dendrimer forms a layer of amino group bearing branched chains extending away from the surface of the slide. The excess Starburst is then washed away and the well is allowed to interact with 1 M Trisma base for a short time to block the remaining sites on the well. The Starburst treated well is then activated by glutaraldehyde, forming Schiff-bases from the amino groups, which is followed by a rinse. The AChE is then allowed to interact with the activated dendrimer for approximately two hours. Two methods of blocking the excess sites on the dendrimers have been used. The first method used was blocking with casein, as before. Concerns about casein interactions with other chemicals to be used have necessitated a new procedure. The excess sites are currently being blocked with TRIS, which is less likely

to interact with porphyrins in a manner similar to AChE. Blocking reduces non-specific interactions of the porphyrin with the Starburst as well as stabilizing the enzyme. The Starburst treated plates show much higher catalytic activity, indicating higher enzyme density, than the previous types and the lifetime has been extended to thirty days when refrigerated, indicating greater stability. The catalytic lifetime of the wells showed no pH dependence when stored from pH 5 to 9.

3.2.2 FINAL MODIFICATIONS

The wells themselves presented some problems. The wells are of clear plastic with dimensions that are inconvenient, 7 mm diameter cylinder with height 10 mm, which restricts their measurement in a spectrophotometer. The new binding method offers some concern as to the proximity of the sides of the wells. There is a possibility of binding the enzyme to the sides rather than the bottom making measurements more difficult. The small size of the surface was also of some concern in that we would be trying to measure spectral changes with a very small number of enzymes present. The available number of enzymes strongly effects the detection limit. The less enzymes which are effected, the smaller the absorbance change, so with more enzymes present on the surface the lower limit of detection can be reduced. The small number of particles would also limit the reusability of the surface. Reusability in this case refers to the fact that not all enzymes are effected upon exposure to an inhibitor. These intact enzyme-porphyrin complexes can be effected by subsequent exposure to inhibitors. All of these difficulties could be alleviated through the use of a flat surface.

The immobilization technique previously described is applied to ProbeOn Plus microscope slides from FisherBiotech (Pittsburgh, PA) with very similar results.

ProbeOn Plus slides have a prepared surface so that activation with glutaraldehyde forms Schiff bases from the present amino groups which can subsequently bind other amino groups. The catalytic lifetime is approximately thirty days for casein-blocked slides and somewhat less for TRIS-blocked slides. Catalytic activity cannot be measured using the Ellman procedure after exposure to $TPPS_1$. Preliminary studies, however, show that $TPPS_1$ extends the lifetime of the immobilized AChE if it is applied before storage. The slides show high enzyme density across the larger surface area. The use of glass instead of plastic allows for application with different types of spectrometers and resistance to damage by different environments.

The density of the bound AChE can be increased by using the glutaraldehyde-starburst sequence twice. Catalytic activity studies indicate that they are less stable than slides treated only once. It may be possible to extend the usable lifetime of these slides by treating with $TPPS_1$ prior to storage. Activity is maintained at higher levels for longer periods of time when the slides are stored refrigerated in buffer. Further testing of these slides will be required.

CHAPTER 4

The ultimate goal of this project is to develop a real time detector for acetylcholinesterase inhibitors, organophosphates, nerve gases, drugs, and pesticides, in air and water. Preliminary work has been done using the immobilized AChE with $TPPS_1$ and inhibitors in solution. To understand how the immobilized enzyme performs as compared to the enzyme in solution, different methods of exposure to $TPPS_1$ have been employed as well as different methods of exposure to inhibitors and to substrate.

4.1 PRELIMINARY RESULTS

4.1.1 MEASUREMENT TECHNIQUE

Preliminary data has been collected in the form of absorbance spectra generated through the use of the experimental setup shown in Figure 4.1. Absorbance spectra were collected using a dual wavelength spectrophotometer (SDB - 3 Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA). The output of the spectrophotometer was focused onto the round end of a $^1/_8$" optical fiber bundle that terminates in a linear array (circular to linear bundle; Dolan-Jenner). The microscope slide (n = 1.5151) is butted against the linear array such that the light enters the plane of the glass. On the opposite side of the slide (1" distance), the slide butts against another linear to circular bundle to gather the light transmitted through and evanescently along the slide surface (*68-71*). The circular end of the bundle is then placed for maximal illumination of the R928 photomultiplier photocathode (Figure 4.1).

4.1.2 METHODS OF EXPOSURE TO $TPPS_1$

In order to bind $TPPS_1$ to the active site of immobilized AChE, 200 μL of a 74 μM $TPPS_1$ solution is then placed on the slide and allowed to interact for 15 minutes. The excess is then washed away with 50 mM pH 7 sodium phosphate buffer. It has been determined that this method is preferable to allowing the $TPPS_1$ solution to dry on the slide. As the solution dries, the $TPPS_1$ dries in a high concentration that causes stacking of $TPPS_1$ and distorts the interaction with other chemicals (*3*). Exposure to AChE inhibitors and substrates is achieved in a similar manner, with the concentrations in solution as desired.

4.1.3 EARLY DATA

The absorbance spectrum of a typical AChE-immobilized slide treated with $TPPS_1$ is shown in Figure 4.3 (Trace 1); addition of acetylcholine iodide results in the spectrum shown in Figure 4.3 Trace 2. Figure 4.3 Trace 3, the difference in the spectra of $TPPS_1$ bound to immobilized AChE in the presence and absence of substrate, shows the absorbance change at 446 nm. The peak at 446 nm represents the $TPPS_1$ interaction with the immobilized enzyme. Peak assignments were determined in two ways. First, the peak at 446 nm is not observed when $TPPS_1$ is added to a slide which has been through the immobilization procedure without AChE (Figure 4.2). Second, the spectral changes observed upon exposing the AChE-$TPPS_1$ slide to AChI are only at 446 nm (Figure 4.3, Trace 3).

As described previously (*72*), the addition of tetracaine to the $TPPS_1$-AChE complex in solution results in the expulsion of the $TPPS_1$ from the active site which is observable as a spectral change where the characteristic peak of AChE-$TPPS_1$ interaction (445 nm) disappears. Similar experiments were preformed with AChE immobilized

slides. Addition of tetracaine, a competitive inhibitor of AChE, should, as observed in solution, displace $TPPS_1$ from the active site, and result in an absorbance decrease at 446 nm (the wavelength of the $TPPS_1$-immobilized AChE complex). As seen in Figure 4.4, a decrease in absorbance at 446 nm is seen in the presence of 3ppb (10 nM) tetracaine in both the absolute (Trace 2) and the difference spectrum (Trace 3).

The decrease in intensity of the 446 nm peak is linearly dependent on tetracaine concentration from 300 ppt (1 nM) to 100 ppb (333 nM) (Figure 4.4, Panel A). When the change in absorbance is plotted versus the log of tetracaine concentration, two slopes are observed as seen in Figure 4.5, Panel B. The limits of detection commonly sought for nerve agents are approximately 2 - 5 micrograms per liter (2-5 ppb). On the basis of Figure 4.5, it appears that 300 ppt may approach the detection limit for this method based on sensitivity of the spectrophotometer.

4.2 FUTURE WORK

As earlier stated, the intent of this project is the ability to detect acetylcholinesterase inhibitors at low levels in several types of samples. The immediate goals will be (1) further development of immobilization techniques in the hope that a higher AChE density can be achieved on the slides and (2) a better understanding of the interactions which take place between the AChE, $TPPS_1$, inhibitors, and substrates in this new type of environment. Higher AChE density, will allow for a larger signal to noise ratio. The greater this ratio the more reliable the readings become and the lower the concentration of inhibitor required for detection. The binding affinity of AChE for $TPPS_1$ must be less than that of AChE for the inhibitor to be detected in order for this

type of sensor to function. Knowledge of the association constants for AChE, its inhibitors, and $TPPS_1$ is important for prediction of sensor performance.

While the optical detection spectrophotometer utilized in this study was a bench mounted unit, the magnitude of the absorbance change is obtainable by smaller less-expensive units, including handheld CCD/diode array based spectrometers. That the absorbance changes are observed at specific wavelengths also allows for the use of small solid-state photodiodes fitted with bandpass filters at the appropriate wavelengths. A portable unit, utilizing these types of components is planned to facilitate application in the field. Cost for one slide, excluding labor, is less than five dollars. It is likely that a portable unit for measurement can be designed for around two hundred dollars.

EXPERIMENTAL SETUP FOR SLIDE MEASUREMENTS

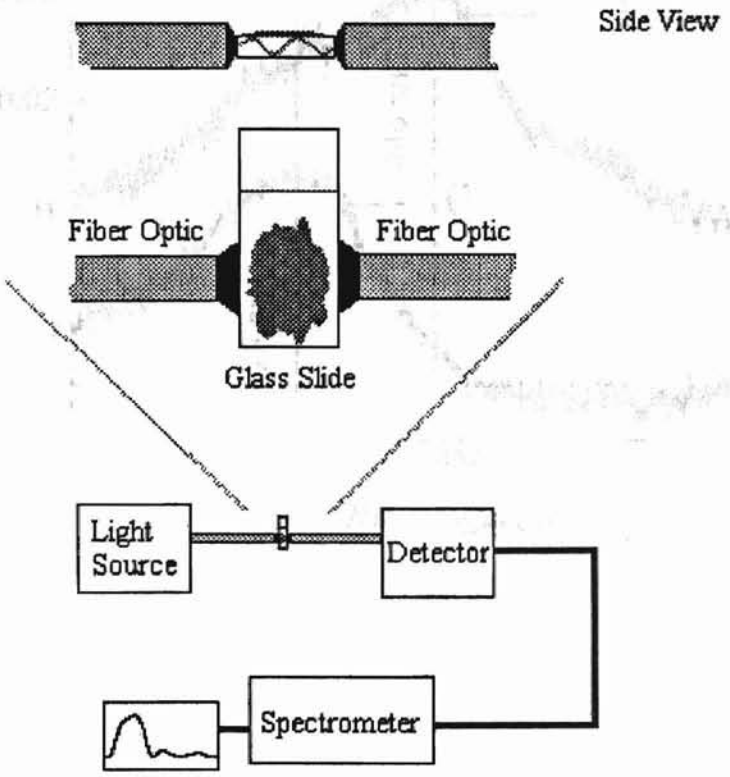

FIGURE 4.1 The experimental setup for measurement of immobilized enzymes on ProbeOn slides uses a fiber optic light source and measures absorbance through the use of the evanescent wave.

ABSORBANCE SPECTRA FOR $TPPS_1$ IN THE ABSENCE / PRESENCE OF IMMOBILIZED ACHE

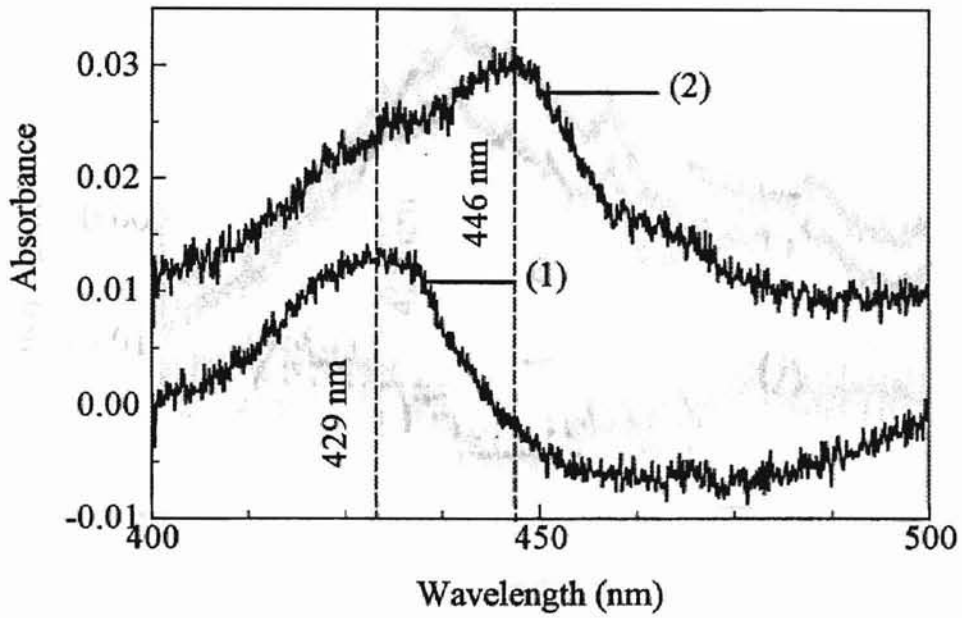

FIGURE 4.2 Trace 1 shows $TPPS_1$ on a ProbeOn slide. The porphyrin is not covalently bound, it is held only by weak interactions. Trace 2 shows $TPPS_1$ on a ProbeOn slide which holds immobilized AChE. Both interactions with the AChE and with the slide result in this case.

EFFECT OF EXPOSURE TO ACHI ON IMMOBILIZED COMPLEX

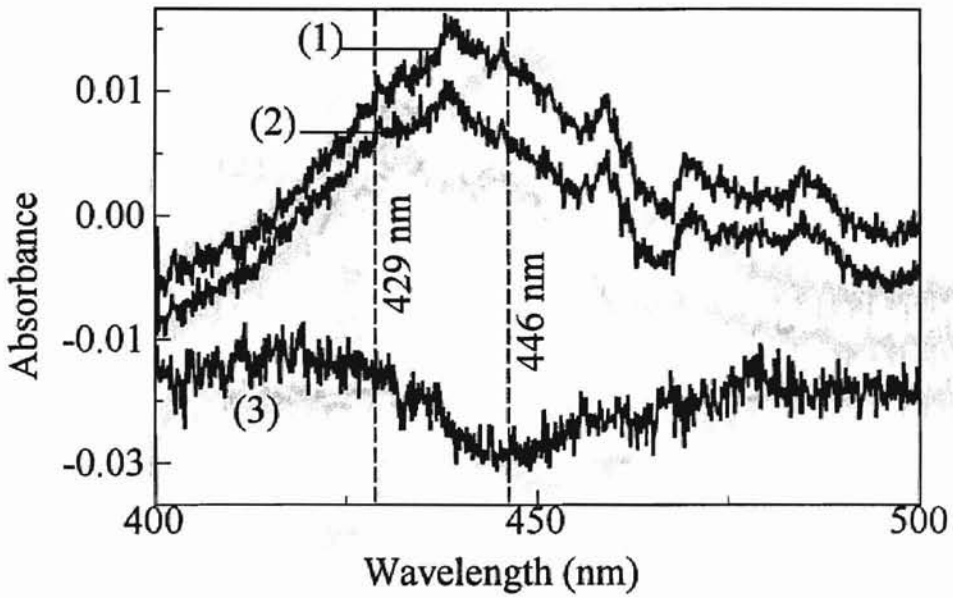

FIGURE 4.3 Exposure of the immobilized $TPPS_1$-AChE complex (Trace 1) to AChI results in expulsion of $TPPS_1$ from the active site of AChE (Trace 2). This is seen in the difference spectrum, $TPPS_1$ + AChE + AChI minus $TPPS_1$ + AChE as a loss at 446 nm.

SPECTRAL CHANGES UPON EXPOSURE TO TETRACAINE

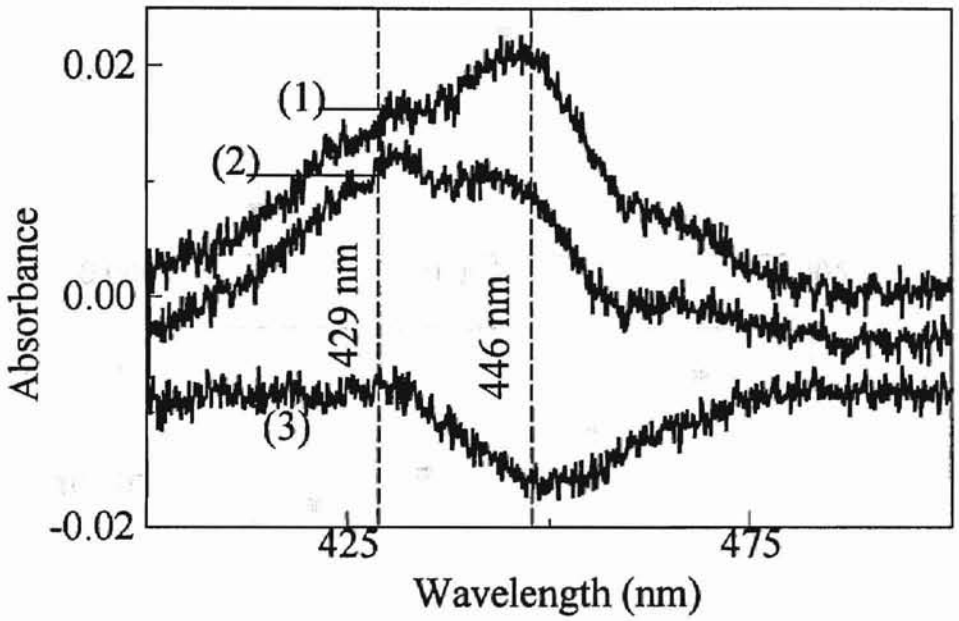

FIGURE 4.4 Exposure of the immobilized AChE-$TPPS_1$ complex (Trace 1) to tetracaine (3 ppb) causes expulsion of $TPPS_1$ from the active site of AChE (Trace 2). This is seen as a loss in the peak at 446 nm in the difference spectrum $TPPS_1$ + AChE + tetracaine minus $TPPS_1$ + AChE (Trace 3).

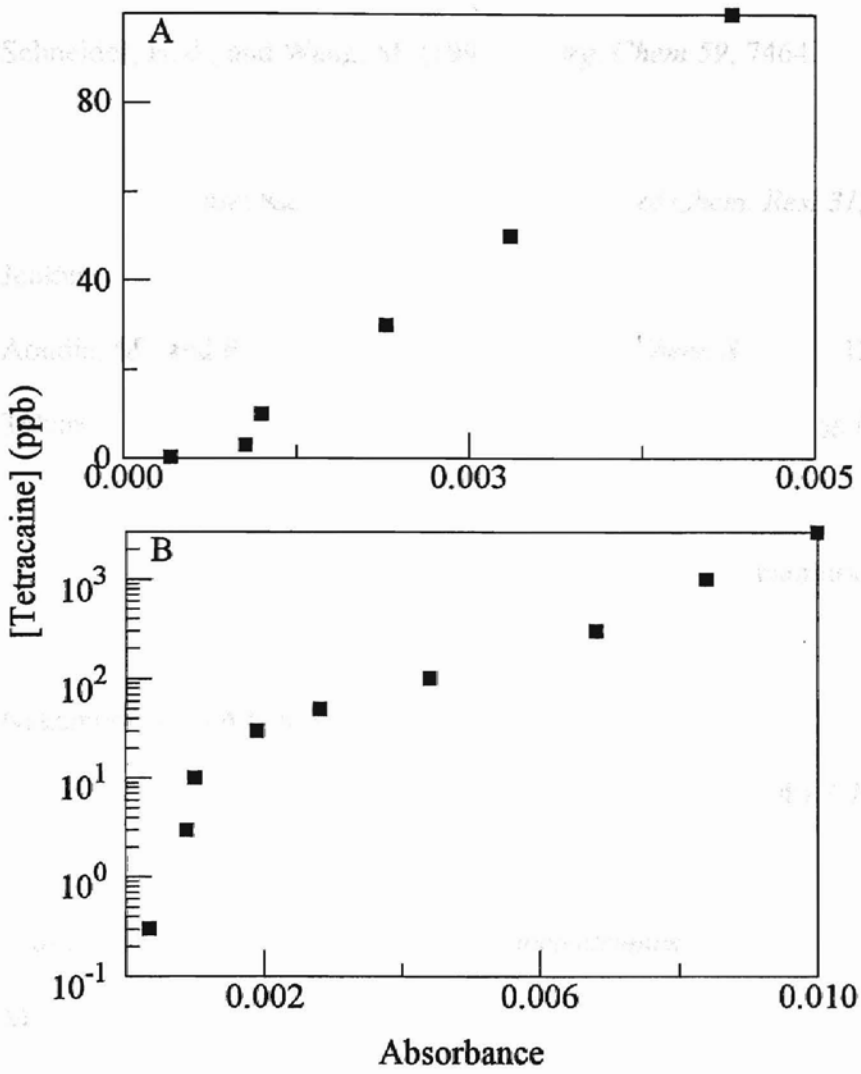

FIGURE 4.5 The dependence on tetracaine concentration of the 446 nm absorbance change. Panel A. Change in absorbance versus tetracaine concentration. Panel B. Change in absorbance versus the log of tetracaine concentration.

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