



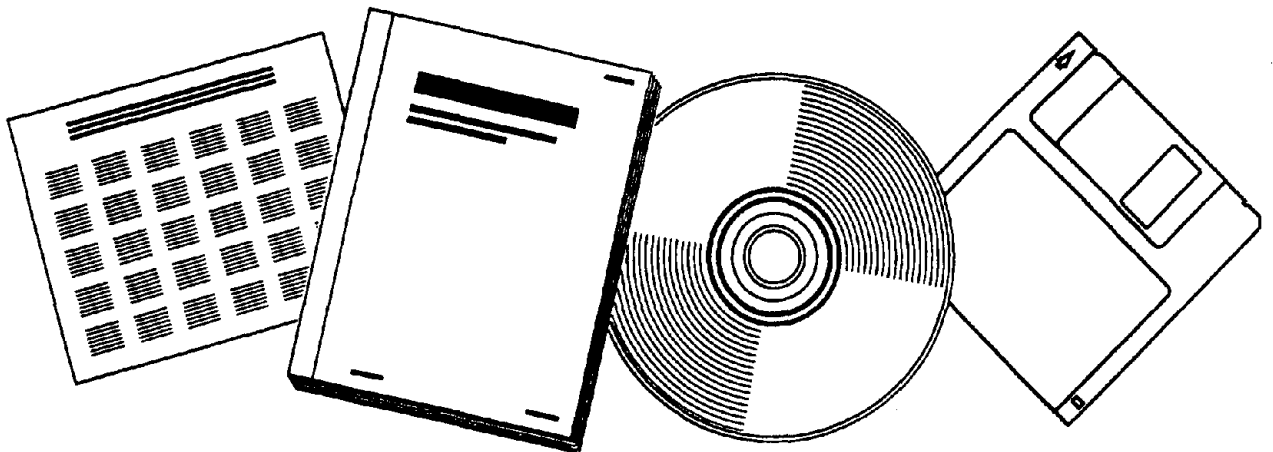
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WESTERN BLOT ANALYSIS OF ORGANOPHOSPHATE EXPOSURE

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16. Abstract (Limit: 200 words) A novel Western blot assay was developed which could detect acetylcholinesterase (AChE) from human erythrocytes. Isoelectric focusing (IEF) was used to separate at least five major isozymes of AChE. Inactivation of the catalytic activity with organophosphate pesticides did not change the isoelectric point of the isozymes, and therefore separation of native from organophosphate inhibited AChE did not occur. The degree of organophosphate inhibition did not influence the recognition of AChE by the monoclonal murine HR anti-AChEs. Native and organophosphate inactivated AChEs were not resolved by Western blotting that used alternative buffering systems. Treatment with organophosphates, diisopropylphosphorofluoridate (55914) and paraoxon (311455) produced aged AChE as evidence by resistance to reactivation with pralidoximine. The authors conclude that the charge difference between native and organophosphorus treated and aged intact AChEs is not sufficient for separation by IEF or electrophoresis. However, an internally controlled protein based assay would eliminate both interindividual and intraindividual variability, thus making it possible to assess AChE inhibition in the absence of preexposure values.					
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Abbreviations

Ab	antibody
AChE	acetylcholinesterase
BCIP	bromochloroindoyl phosphate
DIPFP	diisopropylphosphofluoridate
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
Hb	hemoglobin
IEF	isoelectric focusing
MOPS	3-[N-morpholino]propanesulfonic acid
NBT	nitro blue tetrazolium
OP	organophosphate
PAGE	polyacrylamide gel
PAM	pralidoximine
pI	isoelectric point
PVDF	polyvinylidene difluoride
SDS	sodium dodecylsulfate
TEA	triethanolamine

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Significant Findings

1. A novel Western blot assay capable of detecting acetylcholinesterase (AChE; EC 3.1.1.7) from human erythrocytes was developed. This method uses isoelectric focusing to separate at least 5 major isozymes of AChE. However, inactivation of the catalytic activity with organophosphate pesticides did not alter the isoelectric point of the isozymes and so separation of native from organophosphate (OP)-inhibited AChE was not achieved.
2. Recognition of AChE by monoclonal murine HR anti-AChEs was not influenced by the degree of OP inhibition.
3. A conventional Western blot assay using electrophoretic separation was developed to detect AChE.
4. Western blotting that used alternative buffering systems for electrophoresis did not resolve native and OP-inactivated AChEs.
5. Treatment with the OPs, diisopropylphosphorofluoridate (DIPFP) and paraoxon, produced aged AChE as evidenced by resistance to reactivation with pralidoximine (PAM).

Usefulness of findings

The IN Western blot assay that we present in this report is not currently suitable for workplace monitoring. However, we believe that developing a biological monitoring method to concurrently detect inhibited and active AChE is an achievable goal. An internally controlled assay would have many advantages over the current approach which is based on the measurement of AChE activity. Detection of both inhibited and native AChE in a given individual at a single point in time would eliminate the effect of interindividual variability making it possible to measure OP exposure without prior baseline determinations. Such an assay would also eliminate the limitations in detection limits arising from intraindividual variability which is about 10% (Yager et al., 1976). Interference due to hemoglobin (McConnell et al., 1992; Wilson et al., 1996) would also be eliminated by approaches based on protein separation.

Our experience in developing the IN assay will be useful when others are attempting to develop protein-based assays AChE. The proposal was straightforward. We sought to detect two species of the enzyme, inhibited/aged AChE and native AChE. This was to be accomplished by exploiting the physicochemical differences that occur when OPs interact with AChE. Specifically, aging of AChE produces alterations in the charge and molecular weight when the catalytic serine is phosphorylated. Aging of AChE has been observed after treatment with the OPs, DIPFP and paraoxon (Segall et al., 1993) and our studies suggested that aging had occurred. Finally, the techniques used to separate the phosphorylated from the native protein were methods appropriate for separating proteins on the basis of charge differences.

The success of the IN Western blot assay was contingent upon the separation of the native from aged AChE protein. Separation was to be followed by immunochemical detection that recognized both protein species similarly. Investigators attempting assays based on the separation of proteins will need to circumvent the apparent absence of physicochemical changes in OP-treated but intact AChE. It is possible that the charge is internalized and protein denaturation will be required.

Rather than separating AChEs, alternative approaches could focus on detection methods that discriminate between the native and aged enzyme. For instance, antibodies directed toward epitopes in the catalytic region, particularly the phosphorylated serine, would be useful. A disadvantage of this approach is the alkyl groups remaining after aging will vary with different OPs making it difficult to generate a universal OP-AChE antibody.

The general approach of using quantitative Western blots that substitute isoelectric focusing for electrophoresis would be useful in biological monitoring for other endpoints such as the detection of glycosylated proteins, adducted proteins or enzyme polymorphisms associated with susceptibility. Western blots employing isoelectric focusing are a particularly attractive analytical approach to routine biological surveillance. Isoelectric focusing is a high resolution method capable of separating protein with pI's that differ by as little as 0.02 pH units (Garfin, 1990). In most instances, it should be possible to perform assays with μ l quantities of blood and little sample preparation.

Abstract

Illness due to exposure to organophosphate pesticides has been an persistent problem among agricultural workers. The general population is also at risk of organophosphate (OP) pesticide-induced illness through the consumption of agricultural products, contact with environmental residues (Richter et al., 1992) and accidental ingestion, particularly among children (Levine and Doull, 1992). Measurement of erythrocyte acetylcholinesterase (AChE; EC 3.1.1.7) can be used to establish whether putative cholinergic symptoms are due to OP exposure. However, the greater than 2-fold variability in normal levels of AChE activity makes it difficult to interpret the results in the absence of baseline measurements of AChE activity.

The purpose of this project was to develop an assay that would measure AChE protein rather than enzyme activity. This was to be accomplished with a Western blot procedure where the OP-native and inhibited AChE would be separated by electrophoresis. After separation, the quantities of inhibited/aged and native AChE would be measured with anti-AChE antibodies. The ability to compare inhibited/aged and native AChE would make it possible to screen populations that have not undergone preexposure monitoring.

The development of this method has two requirements: First, the native and inhibited/aged AChE must be separated. Second, the specificity of the anti-AChE should not be influenced by OP-inhibition.

Electrophoresis was chosen to separate AChEs on the basis of the charge difference that accompanies aging. AChE was inhibited *in vitro* with the OP, diisopropylphosphorofluoridate (DIPFP). Separation of AChE was to be accomplished with buffers having pHs similar to the pI of native AChE (5.2 to 5.7). Under these conditions, the native AChE would remain stationary whereas the inhibited OP, with its slightly lower pI, would continue to move through the gel. However, the very limited migration of the AChE during electrophoresis precluded a detectable separation of the native and inhibited AChEs. A different separatory method, isoelectric focusing (IEF), was found to be superior as evidenced by the resolution of the isozymes of native AChE. Using this more appropriate method, we found that treatment of AChEs with DIPFP or paraoxon, the neurotoxic metabolite of parathion, did not produce a change in the isoelectric point as evidenced by a shift in one or more of the focused bands. Experiments with pralidoximine, which reverses inhibition in unaged enzyme, showed that most of the inhibited AChE preparations were aged.

Immunodetection of Western blotted native and inhibited AChEs indicated that antibody recognition was not influenced by the treatment of the AChE with OPs.

We conclude from these experiments that the charge difference between native and OP-treated and aged intact AChEs is not adequate for separation by IEF or electrophoresis. However, an internally controlled protein based assay would eliminate inter- and intraindividual variability making it possible to assess AChE inhibition in the absence of

preexposure values. Therefore, our experiments provide direction for future strategies; specifically, separation of the intact from native AChE will require additional steps to exploit the presence of the phosphorylated serine. This could be accomplished by either externalizing the phosphorylated serine by denaturating or by derivatization. An alternative to separating the native from inhibited AChE would be to develop antibodies that distinguish epitopes on the basis of phosphorylation.

Background

The problem

Organophosphate pesticides are widely used pesticides that have been historically associated with acute toxicity and continue to pose a significant hazard to agricultural workers. A recent study performed by Cal EPA found that the risk of severe illness was related to exposure to organophosphate residues, and jobs that involve mixing, loading, or application of organophosphate pesticides (Weinbaum et al., 1995).

Organophosphate pesticides are potent neurotoxicants which act by inhibiting the activity of cholinesterases, enzymes which degrade the neurotransmitters acetylcholine.

The inability to degrade acetylcholine results in accumulation of the neurotransmitter and overstimulation of nerve cells. OP inhibition of peripheral nervous system causes cholinergic symptoms of sweating, salivation, miosis, and incontinence while central nervous system inhibition can result in psychosis and unconsciousness (Morgan, 1980). Incidents of acute poisoning have been implicated in chronic neuropsychological disturbances (Rosenstock et al., 1991).

Biological monitoring of exposure can be accomplished by measuring cholinesterase activity in either the serum as butylcholinesterase or in erythrocytes as AChE. AChE is an integral erythrocyte membrane protein whose function is unknown. AChE is preferred for biological monitoring because, unlike butylcholinesterase, it corresponds the form of cholinesterase in the central nervous system (Coye et al., 1986) and its activity is not influenced by metabolic factors such as alterations in liver function (Brewster et al., 1992; Trundle and Marcial, 1988).

Measurement of AChE activity has been extensively used to monitor exposure in agricultural workers and to diagnose OP poisoning in clinical settings (Midtling et al., 1985). The state of California requires medical surveillance of AChE levels when workers use OP pesticides more than 30 hours in a 30 day period (CDFA, 1982).

Limitations of current methods

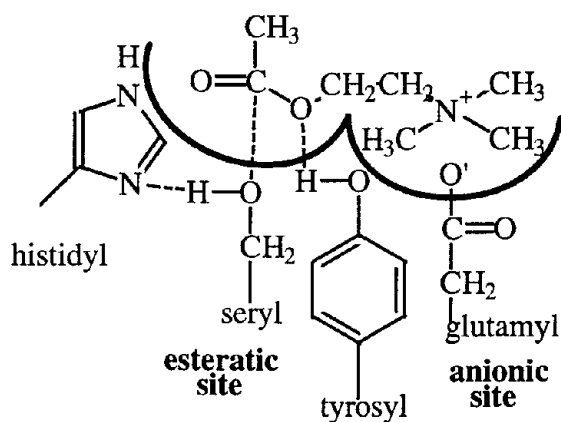
Approaches for using AChE in biological monitoring are based on measurements of catalytic activity. Many different techniques have been used to measure AChE activity (Wilson and Henderson, 1992). The simplest and most widely used assay is a colorimetric method based on a reagent known as Ellman's reagent, (5,5'-dithio (2-nitrobenzoic acid) (DTNB)). The assay measures AChE activity in the presence of the substrate acetylthiocholine which is hydrolyzed into thiocholine and acetate. After reduction by thiocholine, Ellman's reagent can be detected at 412 nm (CalEPA, 1994; Magnotti et al., 1988). While the assay has been shown to be reproducible, there is considerable intraindividual (~10%) and interindividual (>100%) variability in rigorously measured normal AChE levels (Yager et al., 1976; Ames et al., 1989; Rosenstock et al., 1990). AChE activities are meaningful only when compared to activity measured in the absence of exposure, especially in situations where exposure is low. Occupational biological monitoring is effective because of preexposure activity

measurements, an option not available in situations where exposure is accidental or unintentional.

Addressing the problem with an internally controlled assay for AChE proteins

The "IN assay" we proposed would assess AChE as protein rather than measuring catalytic activity. Native and inhibited AChE proteins separated by electrophoresis would be immunochemically detected with a monoclonal anti AChE which recognizes an epitope that is not affected by the inhibition. By concurrently measuring inhibited/aged and native AChE protein, the assay can be interpreted without preexposure screening. In addition, assays that measure AChE proteins eliminate the effect of intraindividual variability making it possible to assess smaller degrees of inhibition.

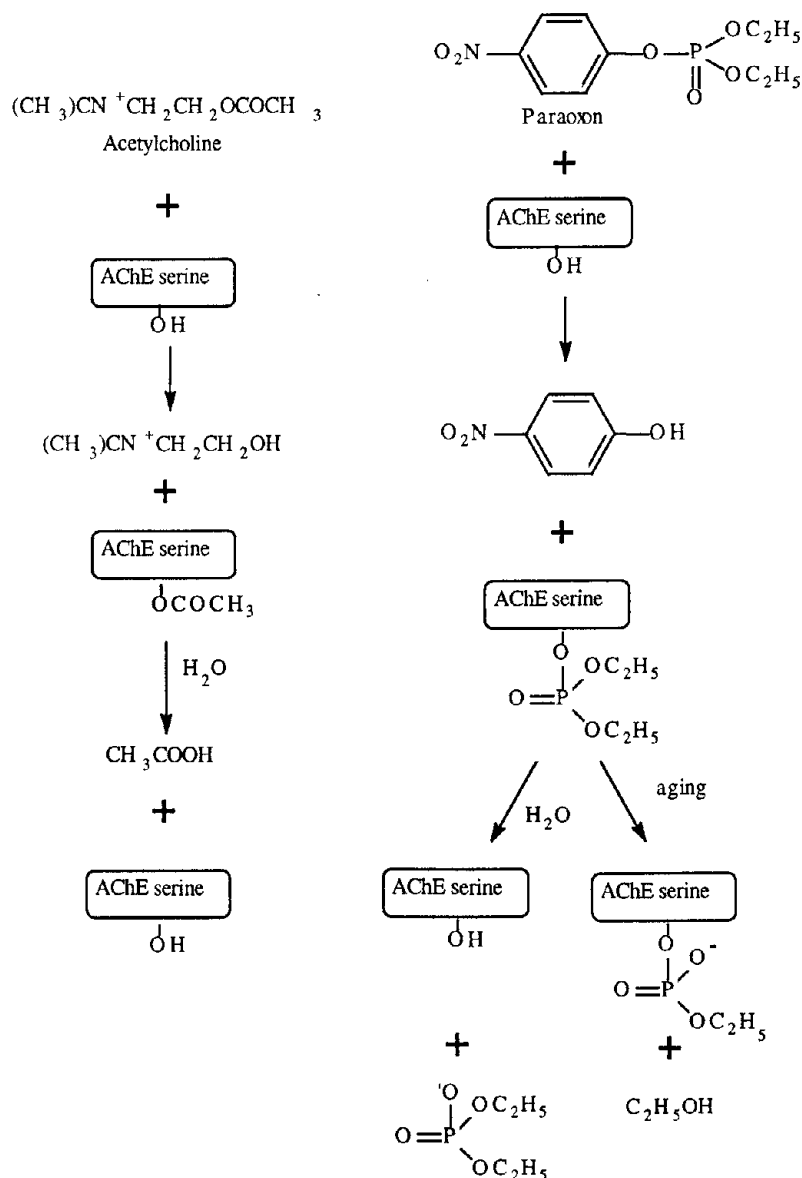
Separation of native and inhibited AChE should be feasible based on what is known about the charge alterations that accompany enzyme inhibition. Substrate interacts with AChE at two distinct sites. One is the anionic site which attracts positively charged moieties such as quaternary nitrogens. Catalysis takes place in the esteratic site. Like all serine hydrolases, the activity of this site is conferred by three amino acids, serine, glutamic acid and histidine. Organophosphate pesticides occupy the esteratic site and unlike endogenous substrates, are not rapidly turned over. Instead, the OP inhibits enzyme by physically preventing interaction between the catalytic serine with the substrate acetyl group.



Initially, treatment with pyridinium oximes such as pralidoxime can remove the OP from the site and reactivate the AChE (Jokanovic et al., 1996). Irreversible inhibition occurs when one of the alkyl moieties of the OP is hydrolyzed. After dealkylation, the enzyme is irreversibly phosphorylated at the catalytic esteratic site. This irreversibly inhibited AChE is referred to as "aged" and has an additional negative charge due to the phosphorylation. The acquisition of the negative charge that accompanies aging of OP inhibited AChEs has been confirmed by ³¹P NMR (Segall et al., 1993).

The negative charge acquired during aging is the basis for the separation. Separatory methods such as electrophoresis and isoelectric focusing routinely separate isozymes (Qin Wei and Xue, 1991) and proteins that differ only by the presence of a single phosphorylated site (Arigoni et al., 1996; Nakamura et al., 1995). Furthermore, a single

known genetic variant of AChE called the YT blood group antigen substitutes a asparagine for histidine at codon 322 which does not affect separation by IEF (Masson et al., 1994).



Assays that exploit the charge difference produced by aging should separate native and reversibly inhibited AChEs from irreversibly inhibited AChE. This contrasts with assays of activity which measure the effect of both reversible and irreversible inhibition. This subtle difference is significant since the protein assay would measure irreversible inhibition that is not subject to medical intervention. The ability to measure the proportion of aged enzyme would be useful in investigating questions regarding the health effects of low level exposure. In particular, an assay detecting AChE protein

would be useful in examining relationship between aged enzyme and the persistent effects following chronic exposure and poisoning incidents (Rosenstock et al., 1991; Savage et al., 1988).

Strategy for developing an assay to measure inhibited and native AChEs

The intent of the project was to use charge difference as the basis of separation. This was to be accomplished by electrophoresis with buffers with pHs that matches the isoelectric point of the native AChE. AChE has an isoelectric point of 5.7 (Fritze and Beckmann, 1987) and an additional negative charge should reduce the isoelectric point. In buffers having a pH of 5.7-5.8, the native AChE should be immobile while the inhibited and aged AChE should continue to migrate slowly through the gel.

The overall goal was to develop a Western blot method, the IN assay, that exploited the difference in charge produced by aging to separate irreversibly inhibited from native enzyme and to use anti-AChE antibodies for detection as shown in Table 1 steps I-V. The IN assay would be optimized and characterized in steps VI to IX. Finally, the IN assay was to be used to measure exposure in OP exposed workers and the results compared to those obtained by measuring activity with Ellman's reagent.

Experiments I and II, the optimization of the electrophoresis and blotting, used native enzyme which was detected with an insoluble histochemical stain for AChE activity. This was necessary to conserve limited quantities of the HR anti-AChEs. Once the methods for blotting the AChE enzyme to the membrane were worked out, experiments to separate native from inactivated AChE with immunochemical detection commenced. The remaining time spent on development focused on the technical aspects of achieving the separation.

Table 1. Summary of proposed experiments

From Proposal				
#	Experiment	Objective	Approach	Outcome
I	electrophoresis	verify mobility of AChE	detect with histochemical stain	accomplished
II	blotting	verify transfer of AChE from gel to membrane	detect with histochemical stain	accomplished
III	anti-human AChEs	determine effect of enzyme inhibition on antibody recognition	compare IC50 values obtained with inhibited and native AChEs	immunochemical detection does not suggest recognition based on activity; see Figure 3
IV	Western dot blot	develop antibody detection system using AChE-spiked blots	determine lowest % inhibited enzyme detectable	in initial experiments, AChE was not detectable either on dot blot or on 96 well plates. Since this step was not critical to assay development we later evaluated the antibodies after the Western blot transfer had been developed.
V	separate native and inhibited enzyme	lower pH of buffer until native and aged enzymes separate	detect separation with antibody detection system	experiments evaluated variables relevant for the separation of AChEs including the use of alternative buffers and an alternative separatory method (IEF)
VI	IN assay	optimize procedures	establish detectable lowest detectable inhibition	
VII	AChE storage for IN assay	determine storage conditions	compare activity of fresh and stored AChEs	
VIII	effect of 5 OPs on IN assay	measure inhibition in AChE incubated with DIPFP, malaoxon, paraoxon, mevinphos, oxydemeton methyl	compare results obtained with IN and Ellman's assays	experiments were performed with DIPFP and paraoxon
IX	variability in the IN assay	measure inhibition, variability in treated blood from cohort	measure variability in IN and Ellman's assay using baseline or weekly references	
X	IN assay application	measure AChE inhibition in OP-exposed workers	detect exposure with IN assay	

Materials and Methods

A. Reagents and Equipment:

Unless stated otherwise, reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Ampholytes (pH 4.0-6.5; pH 3.5-9.5) and "Clean Gels" for isoelectric focusing were obtained from Pharmacia Biotech. Three murine monoclonal anti-AChE antibodies, HR2, HR3, and HR4, were supplied through the kind donation of Dr. S. Brimijoin of the Mayo Clinic.

Equipment and consumables include Immobilon 2 96-well plates; Mini-Protean II electrophoresis cell with an electrophoretic transfer cell, polyvinylidene difluoride (PVDF) membrane from Biorad (Richmond, CA); Immobilon P membrane from Millipore (Bedford, MA); Pharmacia isoelectric focusing apparatus, power supply and gel cutter (Uppsala, Sweden); Molecular Devices densitometer with Imagequant software; Molecular Devices plate reader (Sunnyvale, CA).

B. AChE preparations

a. Commercial human erythrocyte AChE (Sigma Lot 81H9333) This was a crude extract with a specified AChE activity of 50 U/200 mg. For use, AChE was prepared by dissolving 1.5 mg of the powder in 0.5 ml of Tris buffer (pH 7.5) with 0.2-1% Triton X-100.

b. Erythrocyte lysate Erythrocytes from blood collected in heparin tubes were allowed to separate by settling for several hours. After the plasma and buffy coat were decanted, the erythrocytes were centrifuged for 10 minutes (5000 x g) and the remaining serum was removed. 600 µl of 20 mM Tris buffer (pH 8.0) was added to 400 µl of packed erythrocytes. Cell membranes were solubilized by the addition of 0.2-1% Triton X-100 in dH₂O and overnight incubation at 4°C. Membrane debris was separated by centrifugation (5000 x g) and the lysate was transferred to a clean Eppendorf tube.

c. Erythrocyte ghosts The preparation of erythrocyte ghosts was adapted from the method of Toutant et al, 1989. 1 ml of packed erythrocytes was incubated overnight at 4°C in 4 ml of cold distilled deionized water. The next day, the erythrocyte membranes were separated by centrifugation at 4°C. They were washed 4 times in 0.85 NaCl with 15 mM Na₂HPO₄. A second lysis was done by adding 1 ml 4°C water and refrigerating 2 additional hours. Membranes were separated by centrifugation (5000 x g) and washed with NaCl with 15 mM Na₂HPO₄ as before. The pellet consisting of erythrocyte membrane ghosts was brought to a final volume of 1 ml in 20 mM Tris buffer (pH 7.5). During these procedures, ghosts were kept on ice. After the ghosts were prepared, they were solubilized with 0.2-1% Triton X-100.

d. Papain digested AChE AChE was digested with papain using a procedure adapted from Dutta-Choudhury and Rosenberg (1984) and Rosenberg and Scoggin (1984). After ghosts were prepared, reaction mixes were prepared with 100 µl of ghosts with an estimated 2.5 µg of AChE. Reactions were performed with 26, 260 and 1300 µg of

papain. Edrophonium chloride was also added to a final concentration of 100 μM in the solution. Reactions were performed in 20 mM Tris buffer at pH 7.0. Reactions were incubated at room temperature for 2-3 hours.

e. OP inactivation of AChE AChE preparations were inhibited by the addition of either the organophosphate diisopropylphosphofluoridate (DIPFP, CAS 55-91-4) or with paraoxon, the active metabolite of parathion (CAS 311-45-5). DIPFP is a liquid with a density of 1.05 g/ml. DIPFP stocks with the concentration of 0.3 mM, 2.9 mM and 28.8 mM were made by serial dilution in DMSO. Paraoxon has a density of 1.274 g/ml. Paraoxon stocks of 1, 10 and 100 μM were made by serial dilutions in ethanol.

Prior to solubilization, erythrocyte ghosts from 50 to 100 μl of packed erythrocytes in Tris buffer (20 mM, pH 8.0) were inactivated by the addition of DIPFP to final concentrations of 3 to 400 μM . DMSO was added to the control reactions and the total DMSO never exceeded 10 μl . Total reaction volumes were 1 ml. After approximately 1-2 hours, the ghosts were separated from the DIPFP-containing buffer by centrifugation (5000 x g). Afterwards, the ghosts were washed three times in Tris buffer. Assays for AChE activity were done on 96 well plates using the Cal EPA methodology as described above.

Treatment of solubilized erythrocyte lysate with DIPFP was avoided since unreacted the OP was difficult to remove. Untreated erythrocyte lysates were used for initial experiments establishing the conditions for electrophoresis and blot transfer.

Whole erythrocytes were incubated with DIPFP or paraoxon. This approach permitted the removal of excess OP. This was done after allowing adequate time for inactivation, usually 1-2 hours. The intact erythrocytes were centrifugally separated from the OP-containing buffer and washed. Most experiments treated 100 μl of packed erythrocytes with 300 to 3000 pmoles of DIPFP in a total reaction volume of 1 ml. The treated erythrocytes were then lysed and solubilized using the approach described. The removal of excess OP, made it possible to prepare dilutions of inactivated and native enzyme with the preferred level of activity.

C. ELISA assay

An indirect ELISA was used to assess the specificity of the HR anti-AChE antibodies. AChE (0.1 to 100 $\mu\text{g}/\text{well}$) was used as a coating antigen. AChE was diluted in coating buffer containing 1.59 g Na_2CO_3 , 2.93 g NaHCO_3 and 1.0 ml of 20% NaN_3 per liter (pH 9.6). 100 μl of AChE containing coating buffer was pipetted into each well and the plates were incubated overnight at 4 C. After 3 washes with phosphate buffer with Tween (PBS Tween) (8 g NaCl , 1.15 g Na_2HPO_4 , 0.2 KCl , 0.2 g KH_2PO_4 , 0.5 ml Tween 20 and 1 ml 20% NaN_3 in 1 liter) the plates were blocked with PBS-BSA (1% bovine serum albumin dissolved in PBS Tween, filtered through a Whatman #1 filter) for 30 minutes. 100 μl of PBS Tween containing anti-AChE antibodies diluted 1:500 to 1:3000 were added to each well. The plates were incubated at room temperature for two hours followed by 3 washes with PBS Tween. Goat anti-mouse antibody coupled to alkaline phosphatase (100 μl of a

1:1000 dilution in PBS Tween) was added to each well and the plates were incubated at room temperature for 2 hours. The substrate added to each well was nitrophenyl phosphate dissolved in diethanolamine (DEA) buffer (24.25 ml DEA added to 200 ml dH₂O followed by the addition of 0.25 ml 20 % NaN₃ and 25 mg MgCl₂ • 6 H₂O; adjust pH to 9.8, qs to 250 ml). Plates were read at 412 nm on a 96 well plate reader.

D. Western blot procedures

a. Electrophoresis Gel preparation and electrophoresis follow the procedure outlined in Current Protocols in Molecular Biology (1991). Gel solutions were prepared as follows: 5.0% running gels were prepared with 5.0 ml acrylamide solution (30% acrylamide: 0.8% bisacrylamide), 7.5 ml 1.5 M Tris HCl buffer (pH 8.8), 0.1% Triton X-100 and 17.2 ml water. 7.5% running gels consisted of 7.5 ml acrylamide solution (30% acrylamide: 0.8% bisacrylamide), 7.5 ml 1.5 M Tris HCl (pH 8.8), 0.1% Triton X-100 and 14.7 ml water. Stacking gel solutions were prepared with 1.3 ml acrylamide solution (30% acrylamide: 0.8% bisacrylamide), 2.5 ml 0.5 M Tris HCl (pH 6.8) and 6.1 ml water. After degassing, 20 µl TEMED and 50 µl 10% ammonium persulfate were added to the running gels. 10 µl TEMED and 20 µl 10% ammonium persulfate was used for the running gels. After the addition of the polymerizing reagents, the solutions were transferred immediately into the casting stands. When both running and stacking gels were used, the running gels were poured first and the upper surface was covered with water saturated isobutyl alcohol. After the gel has solidified, the isobutyl alcohol was poured off and the gel surface was washed with the Tris glycine running buffer (pH 8.8). The stacking gel was then poured and the comb was inserted. Gels were allowed to polymerize for at least 45 minutes.

Analyte was diluted 1:3 in sample buffer (2.28 g Tris base, 30 ml glycerol, 1.5 mg bromophenol blue and 0.1% Triton X-100 in 100 ml water, pH 6.8). From 2 to 20 µl of analyte was added to the wells with a gel loading pipette tip.

The running buffer consisted of 4.8 mM Tris base and 38 mM glycine (pH 8.8). The Biorad Mini-Protean electrophoresis unit required about 700 ml of running buffer. Electrophoresis was done at 200 volts, 100 mA and required about an hour.

b. Isoelectric focusing (IEF) Protein separations were performed by isoelectric focusing. Pre-cast "Clean gels" were used because additives, such as Triton X-100 could be incorporated into the gel. Gels were soaked for 4 hours in wide range (pH 3.5-10) for range finding or narrow range (pH 4.0-6.4) ampholytes. Depending on the size of the experiment, full (26 x 11 cm), half (13 x 11 cm) or third (8.5 x 11 cm) gels were rehydrated with 3% ampholytes and 0.05% Triton X-100. Full gels required 10.4 ml of rehydrating solution. Half gels required 5.2 ml while third gels required 3.5 ml of solution. Gels were rehydrated for 6 hours and placed on a rocker table. When ready for use, gels were gently blotted to remove excess liquid and placed on the LKB isoelectric focusing apparatus.

The following protocol was used for focusing:

Table 2. Conditions for isoelectric focusing

Purpose	Voltage (V)	Current (mA)	Power (W)	Time (min.)
prefocus	400	4	2.7	30
sample loading	500	2.7	2.7	30
isoelectric focusing	1000	4.7	4.7	180
band sharpening	1200	4.7	6	15

After prefocusing, from 1-5 μ l of the sample was pipetted directly onto the surface of the gel midway between the electrodes. Power was resumed and the gels were focused using the above protocol. For the final 15 minutes, the voltage was increased to sharpen the resolution of the bands.

c. Blotting Proteins from the gels used for electrophoresis and isoelectric focusing were transferred to membranes with the Biorad electrophoretic transfer cell with slight modifications of the procedure recommended by Millipore and Biorad. Proteins were blotted onto PVDF membrane which had been prewetted in methanol. The membrane, two pieces of filter paper cut to the size of the gel, and the pads were equilibrated in running buffer.

The membranes, filters and pads were assembled in the cassettes along with the gels. Electrophoresis gels were carefully removed from the glass supports whereas isoelectric focusing gels were cut from their backing using a gel cutter. The Biorad Mini-protean II has two cassettes making it possible to blot two gels in a single run. After optimization, the best transfer conditions were found to be 100 Volts (maximal attainable voltage), and 350 mA for about 0.5 hour.

E. Detection methods

a. Detection of protein in gels with Coomassie blue Coomassie blue solutions was used to detect protein in the PAGE gels. This was accomplished by first soaking gels in a fixing solution consisting of 50% methanol, 10% acetic acid for 1-2 hours. Gels were transferred to the Coomassie staining solution (50% methanol, 0.05% Coomassie Brilliant Blue R dissolved in methanol, 10% acetic acid and 40% water) for 6 hours. After staining, the gel was transferred to an aqueous destaining solution containing 5% methanol overnight.

b. Detection of protein on membrane with India Ink Detection of protein on PVDF membranes was performed as follows. PBS with 0.4% Tween (PBS Tween) was used for rinses and as a diluent. India Ink (Pelikan) was diluted to a concentration of 0.1% (v/v) in

the PBS Tween. Each membrane to be stained required 50 ml of the India Ink PBS Tween.

Prior to staining, the membranes were washed 3 times in the PBS Tween for 5 minutes. The membranes were transferred to the India Ink in PBS Tween solution for 3 hours. Excess stain was removed by rinsing in PBS Tween and the membranes were allowed to dry.

c. Detection of cholinesterase activity with Ellman's reagent This assay was used to detect AChE in solution as the free thiols produced upon hydrolysis of the substrate. It is adapted from the approach used by Cal EPA (1994) which in turn is based on the method of Ellman et al., 1961. To each well in a 96 well plate, the following reagents were added in order: 200 μ l of 20 mM Tris buffer (pH 8.0), 0-50 μ l of erythrocyte lysate and 10 μ l of 10 mM Ellman's reagents. The final volume was 250 μ l. After the addition of 10 μ l of 10 mM acetylthiocholine substrate, the absorbance at 412 nm was read at 1-5 minutes (Cal EPA, 1994; Yager et al., 1976).

d. Detection of cholinesterase activity with Karnovsky and Root's histochemical stain A histochemical stain developed by Karnovsky and Roots (1964) was used to detect AChE activity on electrophoresis gels and blotted membranes. This stain detects the thiocholine formed upon the hydrolysis of acetylthiocholine by AChE. Thiocholine reduces the ferricyanide to ferrocyanide which then reacts with the CuSO_4 -citrate complex to form an insoluble brown precipitate also known as Hatchett's brown. The formation of the CuSO_4 -citrate complex is necessary to prevent the direct interaction of the CuSO_4 with the ferricyanide.

After equilibration to pH 6.0 by immersion in 100 mM sodium acetate buffer, gels were transferred to a 2X solution of the histochemical dye made as follows: 40 mg acetylthiocholine iodide was dissolved in 26 ml of 0.1 M sodium acetate buffer (pH 6.0). While stirring, the following were added in order: 2.0 ml sodium citrate (200 mM), 4.0 ml CuSO_4 (60 mM), 4.0 ml water, 4.0 ml $\text{K}_3\text{Fe}(\text{CN})_6$ (10 mM).

AChE was evident as a insoluble brown precipitate. Blocking in PBS containing 3% albumin prior to staining was found augment the color.

e. Detection of cholinesterase activity on isoelectric focusing gels (Haddow and Goldfine's method) The ampholytes in the focusing gels interfered with the Karnovsky and Roots method making it necessary to use an alternative precipitating histochemical stain (Haddow and Goldfine, 1985). 50 ml of stain was prepared as follows. While stirring, 390 mg maleic acid was added to 50 ml H_2O . This was followed by the addition in order of 11.5 g Na_2SO_4 , 25 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 35 mg glycine, and 15 mg MgCl_2 . After the pH was adjusted to 6.5 with NaOH, 15 mg acetylthiocholine iodide substrate was added. Activity was evident as a white precipitate that could easily be viewed by placing the gel against a dark background.

f. Immunodetection of AChEs on membrane The method used for immunodetection is adapted from that described by Harlow and Lane (1988). After blotting, the membranes were rinsed 4 times in PBS. The membranes were blocked by soaking in PBS with 3% BSA for 2 hours with agitation. After blocking, the membranes were washed twice in PBS for 5 minutes.

Three murine monoclonal anti-AChE antibodies were used throughout these experiments. They were supplied through the kind donation of Dr. S. Brimijoin of the Mayo Clinic. The antibodies are designated HR2; HR3 ($K_D = 57 \pm 15$ nM) and HR5 ($K_D = 380 \pm 82$ nM) (Brimijoin and Hammond, 1988; Rakonczay and Brimijoin, 1988). The HR antibodies were diluted 1:2000 to 1:3000 in 5-10 ml of 1% BSA-PBS prior to use.

The membranes were agitated during the incubation with the HR antibody. After the antibody was removed, the membranes were washed three times with PBS with 0.2% Tween. The membrane was incubated for 1 hour with agitation with 5-10 ml of goat anti-mouse antibody (1:1000) coupled to alkaline phosphatase diluted in 1% BSA PBS. Excess antibody was removed by four 5-minute washes with PBS containing 0.2% Tween.

A precipitating substrate, nitroblue tetrazolium/bromochloroindolyl phosphate (NBT/BCIP), was added to the membranes immediately after the washes. The substrate was prepared by adding 66 μ l of NBT and 66 μ l of BCIP to the alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM, pH 9.5). The reaction was terminated at 30 minutes by rinsing the membranes with PBS containing 20 mM EDTA.

Results

A. Detection of native and inactivated AChE with HR antibodies by ELISA

We needed to establish whether the anti-acetylcholinesterase antibodies similarly recognized inhibited and native acetylcholinesterases. Rather than wait until the separation and blotting conditions were worked out, we attempted to measure activities by direct ELISA in a 96-well plate. HR antibodies were diluted 1:2000 to 1:3000 and added to wells coated with a commercial AChE-preparation. Detection of the bound HR antibodies was accomplished with goat anti-mouse antibody (1:1000) coupled to alkaline phosphatase.

Color development in the wells coated with from 0.1 to 100 $\mu\text{g}/\text{well}$ AChE did not differ from the color observed in the wells without AChE. Despite the failure of the immunodetection, AChE activity was detected in comparable quantities of enzyme in solution which suggested that the enzyme was not retained on the surface of the well. Because the detection of AChE in 96 well plates required substantial quantities of antibody but was not applicable to the final assay, we decided to not pursue this approach. Instead, HR reactivity towards native and inhibited enzyme were compared after transferal to membrane by the newly developed Western blot procedures.

B. Development of a Western blot using native AChE

a. Detection of active acetylcholinesterase Ellman's reagent was used to detect AChE activity in commercial AChE preparations, erythrocyte lysates, and ghosts intended for electrophoresis. Figure 1 shows the activity of the commercial preparation and of an erythrocyte lysate prepared from fresh blood. In both, significant changes in color development were not detectable in the absence of either enzyme or substrate. The specific activity of the AChE preparation was found to be 0.014 U/mg protein. The activity of the AChE preparation was 3×10^{-5} per μl whereas the activity of the erythrocyte lysate was 6.0×10^{-4} per μl .

After electrophoresis, the Karnovsky and Roots histochemical stain was used to detect AChE activity in the gels and membranes. Since the product is detected as thiols, there was concern that the high concentration of hemoglobin (Hb) which has two free thiols per molecule would produce an unacceptably high background. This required measuring the effect of protein thiols on apparent AChE activity by performing the assay for AChE with and without substrate. Color development in the absence of the thiols produced by the hydrolysis of the substrate would be attributable to non-AChE-related sources.

AChE activity was measured in erythrocyte lysate, albumin, and the commercial AChE preparation. No color development was demonstrable with the AChE or albumin preparations in the absence of substrate. In contrast, about 66-76% of the initial signal of the erythrocyte lysate is attributable to non-AChE sources as shown in Figure 1B. However, the signal due to AChE can be ascertained after subtraction of the background due to Hb.

b. Electrophoresis Electrophoresis was performed using the commercial AChE preparation, erythrocyte lysate and erythrocyte ghosts. After equilibration to pH 6.0 in 100 mM acetate buffer, AChE activity was reliably detected in the PAGE gels. In all, the intensity of the bands corresponded to the volume of the AChE-containing preparation placed in the well. Typically, at least five volumes from 2-20 μ l were used to verify that the activity was related to the quantity of AChE. Detection of activity indicated that the AChE remained intact during the non-denaturing electrophoresis. Human albumin preparations were used as a negative control. Gels with 7.5% acrylamide gave better resolution than 5% gels.

The bands of AChE activity were found at approximately 0.7 cm into the 6.5 cm gel, indicating that the enzyme was not moving far from the origin. Electrophoresis for up to 3 hours did not significantly increase the migration of the AChE. Longer times resulted in buffer temperatures that damaged the gels.

The effect of Triton X-100 on AChE was explored. AChE is a dimeric protein requiring solubilization to obtain the constituent monomers. Triton X-100 at concentrations from 0 to 4% (v/v) was added to erythrocyte lysate. The results, shown in Figure 2, indicate that the activity and resolution was maintained at concentrations from 0.2-2% with little impact of mobility. The differences in resolution suggested heterogeneity of fully solubilized AChE.

c. Optimization of blotting conditions After establishing that enzymatic activity was maintained throughout non-denaturing electrophoresis, transferal of the AChEs from the gel to the membrane by blotting was attempted. The transfer was monitored by using duplicate gels that had been electrophoresed in parallel; one gel was blotted while the remaining gel was stained for AChE activity as a control.

Table 3 summarizes the experiments. Experiments 1 and 2 showed that while AChE activity was maintained in the control and the blotted gels, activity was not detectable on the Immobilon P membrane. Experiment 3 increased the time for electrophoresis and held the gels overnight in the pH 8.8 electrophoresis buffer. These steps inactivated all AChE activity in the gels and membranes. Coomassie staining of the control gel showed protein to be present at a position corresponding to the AChE activity in Experiment 1 and 2 control gels. The blotted gel had neither AChE activity nor protein. While protein bands were present on the membrane at positions consistent with the positions having AChE activity in the control gel. These results indicate that while the conditions inactivated the AChE, transfer to the membrane may have occurred.

Experiment 4 repeated the transfer conditions (90 mA, 6 hours) of the former experiment without the overnight immersion in the electrophoresis buffer. AChE activity was maintained on the control gel but no activity was found in either the blotted gels or membranes. Again, proteins consistent with the position of AChE were detected on the membrane.

Table 3. Optimization of blotting native AChE after electrophoresis

Experiment	Blotting Conditions			Results			
				Electrophoresis only ¹	Electrophoresis and blotting ¹		
	Voltage (V)	Amperage (mA)	Time (hrs)	Control Gel	Gel	Membrane	Backup membrane
1	29	250-160	3	(+)AChE ²	(-)AChE ²	(-)AChE ²	
2	96	250-160	1.25	(+)AChE ²	(-)AChE ²	(-)AChE ²	
3 ³	30	90-?	6	(+)protein; (-)AChE ²	(-)protein; (-)AChE ²	(-)protein; (-)AChE ²	
4 ³	30	90-?	6	(+)AChE ²	(-)AChE ²	(-)AChE ²	
5	30 ⁴	77-250	6	(+)AChE ²	(-)AChE ²	(-)AChE ²	
6	30 50	77-120 200-?	3.5 2.5	(+) protein ⁵	(-) protein (ND) AChE ⁵	(+) protein; (ND) AChE ⁵	(+) protein; (ND) AChE ⁵
7 ⁶	50	130-214	1.5	(+)protein; (+) AChE	(-)protein; (-)AChE	(+) protein; (-)AChE	(+) protein; (-)AChE
8 ⁷	50	118-101	1.5	(+)protein; (+) AChE ²	(-) protein; (-)AChE ²	(+) protein; (-)AChE	(+) protein; (-)AChE
9 ⁸	50	150-200	0.5	ND	(+)protein; (+) AChE ²	(-)protein; (-)AChE	1st: (+)protein; (+)AChE
	100		2	ND	(-)AChE ²	(-)protein; (-)AChE	2nd: (+)protein; (-)AChE 1st: (+)protein; (-)AChE 2nd: (+)protein; (-)AChE
10 ⁹	50	150-200	2	(+)AChE ²	(-)AChE ²	(+)protein; (+)AChE ⁶	(+)protein; (-)AChE ⁶
11	10-35 ¹⁰	22-131	3.25	(+)AChE ²	(-)AChE ²	(-)AChE ²	(+/-)AChE ²
12 ⁹	50	130-200	1.25	(+)AChE ²	(+/-)AChE ²	(+)AChE ²	(+)AChE ²
13 ⁹	25	50-130	3	(+)AChE ²	(+/-)AChE ²	(+)AChE ²	(+)AChE ²
14 ⁹	55	200-250	0.5	(+)AChE ²	(+/-)AChE ²	(+)AChE ²	(-)AChE ²

Table 3. A summary of the experiments performed to determine optimal blot transfer conditions. Ideally, AChE activity that is proportional to the volume of analyte should be detectable in the control gel which was not blot transferred. Conversely, successful blotting to the membrane means that AChE should not be detectable on the blotted gel. The membrane should display AChE activity that is proportional to the volume of untreated AChE used for electrophoresis. No AChE activity should be detectable on the backup membrane(s).

ND: not done

Bold typeface: indicates that the experiment was successful. AChE was transferred only to the membrane with a minimal amount of material remaining on the gel or being transferred to the backup membrane.

¹ Erythrocyte lysate unless otherwise described. Nondenaturing electrophoresis used 7.5% PAGE gels; Immobilon P (Pharmacia) membrane was used for blotting. AChE activity was determined by staining with Karnovsky's histochemical stain.

² A (+) means that the AChE staining was proportional to the volume of untreated AChE loaded onto the gel. A minimum of 5 different volumes were loaded onto each gel.

³ Analytes: erythrocyte lysate, commercial AChE. Gels were kept overnight in electrophoresis buffer (Tris HCl, pH 8.8) at 4°C.

⁴ Constant voltage

⁵ Gels and membrane were stained for protein activity but not for AChE. Coomassie blue was used for gels; India Ink was used for membranes.

⁶ Gels and membranes were cut in half. One half was stained for AChE activity, the other half was used for protein detection.

⁷ nitrocellulose membrane was substituted for Immobilon P; 20% methanol was added to the blotting buffer. Gels were stained for AChE only

⁸ Both gels were blotted. One cassette was removed after a transfer time of 0.5 hours, the other removed at 2 hours. Three layers of Immobilon P were used.

⁹ Biorad PVDF membrane was substituted for the Immobilon P membrane.

¹⁰ The blot was ramped as follows: 10 V for 30 minutes; 15 V for 30 minutes; 20 V for 30 minutes, 25V for 1 hour, 30 V for 45 minutes; 35 V for 30 minutes.

Two variables may contribute to the failure to transfer active AChE. One was the length of time used for the transfer. The other is the failure to maintain constant voltage during the transfer. Typically, the voltage declines 10-50% during the transfer. Experiment 5 examined this variable by maintaining constant voltage during the course of the transfer. The outcome was identical to Experiment 4 where AChE activity was detected on the control gel but not on either the blotted gels or membranes.

Experiment 6 used a backup membrane to determine whether active AChE was passing through the primary membrane in contact with the gel. This experiment differed from the previous experiments because the gels and membranes were stained the presence of proteins but not for AChE activity. Also, the voltage was increased for the last 2.5 hours of the transfer. More protein was detected on the backup membrane than on the membrane in contact with the gel indicating that under these conditions protein was passing through the membrane.

In an effort to reduce the passage of proteins through the primary membrane, the transfer times were reduced to 1.5 hours in Experiments 7 and 8. In Experiment 8, nitrocellulose was substituted for Immobilon membrane which necessitated the addition of 20% methanol to the transfer buffer.

The modifications resulted in a loss of activity during the blot transfer as evidenced by the presence of AChE activity on the control gels but not on either the transfer gel or membrane. The transfer gel had neither protein nor AChE activity, while protein was present in both membranes despite the absence of detectable AChE activity.

Given that protein continued to be transferred through the primary membrane, the transfer time was varied in Experiment 9. Duplicate gels were subjected to electrophoresis in parallel. They were then blotted separately for 0.5 and 2 hours. Since the second gel was no longer available to control for activity, duplicate dilution series were placed on each gel. After blotting, the gels and membranes were cut in half which permitted the detection of both protein and AChE activity. In addition, two backup membranes were used.

The outcome of this experiment that conditions required for complete transfer off the gel did not result in satisfactory transfer to the primary membrane. When 0.5 hour was used for transfer, AChE activity was found on the gel and on the first backup membrane but not on the intended destination of the primary membrane. The backup membranes also stained intensely for proteins. Increasing the transfers to 2 hours eliminated residual AChE activity on the gels but neither was activity found on any of the membranes. However, the backup but not the primary membranes stained strongly for protein.

Experiment 10 varied the membranes. Biorad PVDF membrane with 0.2 μm pores was substituted for Immobilon P membrane with a 0.45 μm pore size. Transfer times of 2 hours successfully moved active AChE from the gels to the primary membrane. AChE

activity was not detectable on the backup membrane although a large amount of protein was present suggesting that some AChE could be lost.

The voltage was ramped in Experiment 11 to determine whether the passage of proteins through the Immobilon P membrane could be reduced. Active enzyme was detected on the control gel but neither the transfer gel or any the membranes had detectable AChE activity.

Biorad PVDF membrane replaced the Immobilon P membrane in all subsequent experiments. In Experiment 12, 50V for 1.25 hours successfully transferred active enzyme to the primary membrane with a small amount remaining on the gel and a small amount being transferred to the backup membrane.

Experiments 13 and 14 further explored the effects of time and voltage on the transfer of AChE to the Biorad PVDF membrane. Experiment 13 used low voltage (25V) for a long transfer period (3 hours) whereas Experiment 14 used the highest attainable voltage (55V) for 0.5 hours. The best results were obtained in Experiment 14 where active AChE was transferred to the primary membrane. A trace of enzyme remained on the gel while no AChE activity was transferred through to the backup membrane.

Based on these results, subsequent blotting employed the highest attainable voltage for 0.5 hours and used the Biorad PVDF membrane.

C. Antibody recognition of native and OP-inactivated AChE

After the conditions for transferring native AChE to membrane and immunostaining had been established, we needed to determine whether the antibodies interacted similarly with native and OP-inhibited enzyme.

Immunochemical detection of native and inhibited AChE in Western blots was compared for each of the three anti-AChE antibodies, HR2, HR3, and HR5. Comparable quantities of AChE were transferred to the membrane as evidenced by the lack of AChE activity on either the transfer gel or the backup membrane.

Table 4. AChE inhibited by incubating 100 μ l erythrocytes with DIPFP:

	DIPFP (pmoles)	AChE activity
1	0	++
2	600	+
3	1500	+/-
4	5800	-
5	28800	-

Figure 3 shows the results of the experiments with HR2 and HR3. While there was considerable variability in the staining, the intensity does not correlate with the AChE activity of the preparation. Neither did the antibodies show an activity-dependent pattern

of recognition, when used to detect AChE inhibited with paraoxon, as shown in Figure 4. Quantitation of the bands by densitometry, shown in Figure 7, confirms that their intensity does correlate with the quantity of protein but not the measured AChE activity.

D. Separation of native and inhibited AChE by electrophoresis and Western blotting

These experiments used AChE preparations either as erythrocyte ghosts or as lysate from treated whole erythrocytes. Erythrocyte ghosts were useful because they were more easily solubilized than whole erythrocytes. They also provided a simpler system for inhibited AChE with less interference from hemoglobin or other proteins. The dose response curve for a typical reaction is shown in Figure 5.

Lysate made from treated erythrocytes where excess OP had been removed was used in the majority of experiments aimed at separating native from inhibited AChEs. The removal of excess OP made it possible to mix AChE treatment groups without inhibiting untreated AChE with the excess unreacted OP. The ability to perform electrophoresis with uniform quantities of AChE in each well was critical because treatments that caused a shift in mobility could be identified without concern that the disappearance of a band was due to low concentration.

The relative mobility of inhibited and native AChEs were monitored as follows. All treatment series were done in duplicate on each gel. After blot transfer, the membranes were cut in half. AChE activity was determined in one half with Karnovsky and Root's histochemical stain. The other half was immunostained with the anti-AChE antibodies. The gels were also stained for AChE activity which aided in determining whether the blot transfer to the membrane was complete.

Generally, the development of the insoluble brown precipitate in the membrane corresponded to the level of OP treatment or the degree of dilution as is apparent in Figure 6 which shows a drawing of a gel where the DIPFP-treated AChE was stained. Figure 9 permits comparison of AChE activity and AChE protein on blots where IEF rather than electrophoresis was used. However, the strategy is identical. The brown bands in Figure 9A are inversely proportion to the level of OP treatment. In contrast, the intensity of the bands on in Figure 9B do not differ with the level of treatment. This is the expected pattern when anti-AChE recognition is not influenced by activity.

Conditions that resulted in differential mobility of the native and treated AChE were sought. Clearly, electrophoresis with Tris glycine buffers at pH 8.8 did not effectively separate native from either DIPFP- or paraoxon-inhibited enzyme. Figure 4 shows the results that were obtained using the lowest concentration of paraoxon (5 pmoles/100 μ l erythrocytes) that completely inhibited catalytic activity. Mixtures with 75, 50, 25 and 10% activities were made using native and OP-inhibited AChEs. After electrophoresis, bands with different mobilities were not evident. This is also the case when the higher concentration of 50 pmoles paraoxon was used. Densitometry analysis of the membrane confirmed the appearance of the bands.

Several alternative electrophoresis buffering systems, listed in Table 5, were explored for their efficacy in promoting the separation of OP-treated from untreated AChE. Electrophoresis was performed with reverse polarity when pH conditions were below the isoelectric point which ranges from pH 4.2 to 5.7 (Fritze and Beckmann, 1987). Blotting required the higher pH of 8.8 to transfer the protein from the gel to the membrane and so was done with the standard Tris glycine buffer.

Table 5. Alternative electrophoresis buffers

Experiment	Buffer system	pH
1	100 mM sodium acetate	7.0
2	100 mM sodium acetate	5.3
3	190 mM TEA-glycine buffer	7.5
4	25 mM histidine, 30 mM MOPS ¹	5.8

¹ McLellan, 1982

A pH 5.8 buffer consisting of 30 mM MOPS (3[N-morpholino]propanesulfonic acid) buffer with 25 mM histidine (McLellan, 1982) was expected to give the best separation of native from OP-treated AChE.

The pH at which the electrophoresis was performed affected subsequent activity. Initial experiments equilibrated the gels in the blot transfer buffer (pH 8.8) prior to blotting. Immediately after electrophoresis, gels run at pH 5.8 and pH 8.8 showed a similar range and level of AChE activity. In contrast, after 15 hours, the activity in the gel that had been run at pH 5.8 was much lower than the activity in the gel run at pH 8.8. Thereafter, blotting was always performed immediately after the electrophoresis.

The necessity to transfer without equilibration affected the quality of the transfer. When gels electrophoresed at pH 5.8 and at pH 8.8 were handled and blotted identically, active AChE was evident in the membrane in contact with the gel electrophoresed at pH 8.8 whereas the AChE activity was much lower in the membrane in contact with the gel electrophoresed at pH 5.8.

Electrophoresis using the alternative pHs, including the experiments using the buffers at the isoelectric point of AChE failed to separate treated from native AChE. In all, the AChE showed little mobility even when the separation time was increased from 1 to 3 hours. Bands were diffuse whether the AChE was obtained from ghosts or erythrocyte lysate. The use of a stacking gel did not improve the resolution of the native and OP-inhibited enzyme.

In summary, Western blot at either standard or alternative pH's failed to separate native from inhibited enzyme. This failure was judged to be to the lack of mobility of AChE under the low pH electrophoretic conditions. This precluded separation on the basis of changes in pI that accompany aging. These results did not preclude separation on the

basis of differential isoelectric points. Rather, they emphasized the need for an appropriate separatory method, isoelectric focusing.

E. Isoelectric focusing

PAGE gels prepared in the laboratory were used for the initial IEF experiments. However, these gels gave unsatisfactory results due to the nonuniformity of their thickness and the difficulty in handling the very thin gels. Native AChE in adjacent lanes varied in their distance from the cathode due to parallel ridges forming perpendicular to the running axis of the gels. Such discontinuities would make it difficult to distinguishing differences in isoelectric points. Running the gels at lower voltages (100 V for 1 hour; 200 V for 3 hours) and allowing the gels overnight to polymerize did not prevent the rippling. The ridges were inferred to be due to uneven dispersion of the ampholytes since they formed in the absence of applied sample.

Subsequent experiments were performed with pre-cast 'clean gels' which could be rehydrated in the presence of 0.05% Triton X-100 (v/v) and either narrow (pH 4.0-6.5) or wide range (pH 4-10) range ampholytes. Proteins with high isoelectric points migrate toward the cathode (negative electrode) whereas those with low isoelectric points move towards the anode (positive electrode). The distance between the electrodes was 10.5 cm.

The strategy for developing a Western blot using IEF was identical to the one used for optimizing the electrophoresis and blotting. Initial studies monitored native AChE activity to establish that the IEF and blotting were successfully moving and transferring functional enzyme. Successful transfer of AChE to the membrane was followed by immunodetection of native and OP-treated AChEs.

When AChE was focused with gels prepared with the wide range ampholytes, approximately 10 AChE bands formed 6-9 cm from the cathode. Hemoglobin from the sample formed two sharp bands that were observable without further staining 3.6 cm from the cathode. The intensity of the bands corresponded to the sample volume which ranged from 0.25 to 2 μ l of erythrocyte lysate. Increasing the voltage (700 V for 20 for entry, 1500 V for 2.5 hours for focusing, 1700 V for 0.5 hour for fine focusing) improved the resolution of the AChE.

When staining for AChE activity according to the method of Karnovsky and Roots, a high nonspecific background due to the ampholytes was observed. Since equilibration in buffer to remove the ampholytes defocused the AChE and hemoglobin bands, an alternative stain was used (Haddow and Goldfine, 1985).

Table 6 shows the variables examined when blotting the isoelectric focusing gels. In Experiment 1, indistinct bands of AChE activity were detected on the blotted membrane. The position of the AChE activity on the portion of the gel that was not

Table 6. Optimization of Western blotting based on separation of AChE by isoelectric focusing

Experiment	Isoelectric and transfer variables	Results			
		Control: nonblotted gel	Blotted gels and membranes		
		AChE activity?	Protein detected?	AChE activity detected?	Immunochemical detection of AChE?
1		8-8.5 cm	ND	gel: 8-8.5 cm (poor resolution) membrane: no activity	ND
2		8-8.5 cm	Yes (membrane)	ND	ND
3	20% methanol during transfer	8-8.5 cm	ND	gel: 8-8.5 cm membrane: no activity	ND
4	10% methanol during transfer	8-8.5 cm	ND	gel: no activity membrane: no activity	ND
5	Tween 20 substituted for Triton X-100	6.5-8.5 cm	+	gel: negative membrane: trace	ND
6	transfer at pH 8.8:	6.5-8.5 cm	+ (trace) membrane	gel: no activity membrane: no activity	ND
	transfer at pH 5.8 (Mops, His):	6.5-8.5 cm	+ membrane	gel: trace at 8.5 cm membrane: no activity	ND
7	electrophoresis & low current transfer	6.5-8.5 cm	ND	gel: trace membrane: activity	+ HR2 - HR3, - HR5
	isoelectric focusing & low voltage transfer	6.5-8.5 cm	ND	membrane: 6.5-8.5 cm	+ HR2 - HR3, - HR5
8	Separate inhibited from native	ND	ND	gel: no activity membrane: activity	+ detected (no difference in migration)
9	Separate inhibited from native	ND	ND	+ range of activity	+ detected (no difference in migration)

ND: not done

blotted corresponded to the position of the activity on the blotted membrane. However, the quantity of most of the AChE activity remained on the unblotted gel indicating incomplete transfer. AChE activity was not detected on the backup membrane indicating that the AChE was being transferred to the membrane in contact with the gel.

Experiment 2 found that proteins with isoelectric points causing them to equilibrate near the cathode, such as hemoglobin, transferred more efficiently than those with lower isoelectric points, such as AChE.

In an effort to improve the efficiency of the transfer to the membrane, membranes were equilibrated in Tris glycine prior to blotting. Equilibration of gel in Tris glycine was found to result in diffusion of the focused bands.

Experiments 3 and 4 examined the effect of 20% methanol, a concentration which maintained full AChE activity, in facilitating the blot transfer. Methanol in concentrations from 10 to 20% reduced transfer to the membrane since active AChE was found on the control and blotted gels but not on the membrane. Hence, methanol was not used in subsequent experiments.

In Experiment 5, Tween 20 was substituted for Triton X-100 during the gel rehydration and used in the transfer buffer. In unblotted gels, the number of bands increased, their resolution improved and AChE activity was enhanced. However, the bands were not visible on the membrane indicating poor transfer. The use of 2.0% Tween 20 in the transfer buffer prevented the adhesion of protein to the membrane. Based on this experiment, Tween 20 was substituted for Triton X-100 during gel rehydration but was not used in the transfer

Given the lack of success at transferring optimally focused AChE, we decided confirm the optimal pH for blot transfer. Experiment 6 compared the efficiency of transfer with pH 5.7 and 8.8 buffers. Both control gels showed AChE activity at the expected location of 6.5 to 8.5 cm. The gel blotted at pH 5.7 showed a trace of activity while the gel from the pH 8.8 transfer showed no activity suggesting that the AChE had been more completely transferred off the higher pH gel. However, both of the membranes showed only a trace of activity which could have been due to mechanical rather than charge related transfer. Staining for protein showed that protein at the expected position had been transferred in both. The bands for the protein were more pronounced on the reverse side of the membrane which suggested that the transfer current was too high. Thus, while active AChE was not transferred, protein having identical isoelectric points were found on the membrane.

The possibility that enzymatically inactive but immunologically recognizable AChEs had been transferred was explored by proceeding with immunodetection with the HR antibodies. Erythrocyte lysates were subjected either to electrophoresis or IEF followed by blot transfer using slightly altered transfer conditions. Previous transfer conditions of 250 mA for 30 minutes were reduced to 25 mA for 40 minutes followed by 250 mA for 30 minutes.

The results showed that active AChE was successfully transferred to the blots from both the electrophoresis and isoelectric gels. Furthermore, the immunostaining showed that the HR2 antibody, but not the HR3, or HR5 antibodies recognized the transferred AChE. The failure of the HR3 and HR5 antibodies may have been due to technical problems with the BCIP substrate which upon inspection was found to have come out of solution. While the HR2 labeled membrane stained, the color faded within a day. This had not been observed previously. Efforts were taken to resolubilize the substrates.

The next step was to distinguish inhibited from native AChEs based on changes in their isoelectric points. The experiments used AChE from DIPFP-treated erythrocyte lysates that had previously been demonstrated to show a range of activities. These were run in duplicate along with a control made from freshly isolated blood. After blotting, half of the resultant membrane was stained for AChE activity while the other half was immunostained using antibody HR2 at a 1: 2500 dilution. The results, shown in Figure 9A, indicate that the AChE activity detected in the 3 groups correlated with the level of DIPFP treatment while in Figure 9B, the AChE proteins from had identical bands despite their different AChE activities.

To determine whether the expected bands were lost due to degradation of AChE by high concentrations of DIPFP, the experiment was repeated by treating fresh erythrocytes with lower concentrations of DIPFP. Figure 10 compares the bands formed by AChE from erythrocytes treated with DIPFP. 100 μ l of erythrocytes were incubated with 0, 600 or 5800 pmoles of DIPFP and after two hours, the excess DIPFP was removed. Treatment with 600 pmoles resulted in a low but detectable level of AChE activity while 5800 pmoles completely inhibited all activity. Also, a 1:1 mixture of the untreated and fully inhibited AChE was used. Each lane contained equivalent quantities of analyte. When the mobilities of the different AChEs are compared, the 5 major bands in all treatment groups and mixtures had comparable isoelectric points.

One explanation for the lack of differential focusing could be failure to prepare aged enzyme. The charge difference necessary for the separation comes about as a result of dealkylation that accompanies the irreversible phosphorylation. If the enzyme was inhibited but unaged, the isoelectric point would be unaltered. To ensure that the enzymes were adequately aged, erythrocytes were treated with pralidoximine (PAM) which has been shown to reverse inactivation in enzyme that is not irreversibly aged (Hobbiger, 1956). The persistence of inhibition after incubation with large excesses of PAM indicates that aging has occurred. The reemergence of activity after incubation with PAM suggests that at least some of the enzyme is not aged.

The results are shown in Table 7. In this experiment, the inhibition produced by 300 pmoles of DIPFP was not reversed by the addition of a thousand-fold molar excess of PAM added late in the incubation. However, PAM did prevent inhibition when DIPFP was used at concentrations of 12 and 60 pmoles. At these concentrations, the excess of PAM was 5000 and 25000-fold. These results suggest that aging occurs after treatment

with DIPFP and only the addition of large excesses of PAM with very low concentrations of DIPFP prevented aging. When the concentration of DIPFP was equal to or exceeded 300 pmoles, the addition of PAM did not prevent inhibition despite the lower molar excess.

These results indicate that with our system, 300 pmoles of DIPFP per 100 μ l of packed erythrocytes will produce aged AChE as evidenced by absence of catalytic activity after incubation with PAM. However, when irreversibly inhibited AChE prepared using 300 or more pmoles of DIPFP were Western blotted, no differences in mobility were found as seen in Figure 11. In Figure 11A, the bands produced by treatment with 300 pmoles DIPFP do not differ from the untreated controls. Figure 11B shows the separation achieved with 600 pmoles of DIPFP with and without PAM. Again, no difference in the pattern is discernible.

We then evaluated whether the failure to discriminate treatment-related shifts in mobility were influenced by the OP. To do this, the experiments were repeated using paraoxon. Table 8 shows that at the lowest level of paraoxon treatment, 5 pmoles per 100 μ l packed erythrocytes, the addition of 500 nmoles of PAM prevented the formation of inhibited enzyme. With higher treatments the AChE remained inhibited suggesting that the paraoxon treated AChE was aged. Based on the electrophoresis experiments shown in Figure 4A neither the magnitude of the treatment or the proportion of treated AChE influenced the position of the bands. When the same materials were separated by the superior separatory technique, IEF, as shown in Figure 4B, lanes 6 (untreated), 5 (treated with 5 pmoles) and 8 (treated with 50 pmoles) show similar AChE mobilities.

In an effort to reduce the effect of the quaternary structure of AChE, a papain digestion was performed. Ghosts were digested using 3 different concentrations of papain. Determination of activity after electrophoresis showed that none of the treated ghosts, included the sham digested had detectable activity after 2 hours of incubation. However, ghosts that were not subjected to the digestion had detectable activity.

Table 7. Reversibility of DIPFP inhibition of AChE.

pmoles DIPFP ¹	PAM: DIPFP	Activity
	Control	+
	PAM w/o DIPFP ²	+
12	25000	+
12	25000 ²	+
60	5000	+
60	5000 ²	+
300	1000	-
300	1000 ²	-
600	250	-
600	250 ²	-
6000	25	-
6000	25 ²	-

¹ 100 μ l of packed erythrocytes were treated with DIPFP with or without PAM (either 150 or 300 nmoles) for 8 hours. Following hours incubation with the treatment, excess reactants were removed and the erythrocytes were lysed by the addition of 0.2% Tween (v/v).

² 150 or 300 nmoles of PAM was added at the initiation of the 8 hour incubation.

Table 8. Reversibility of paraoxon-induced inhibition in AChE

pmoles paraoxon ¹	PAM:Paraoxon	Activity
0	----	+
0	(500 nmoles) ²	+
5	----	-
5	5000 ²	+
50	----	-
50	500 ²	-
500	----	-
500	50 ²	-

¹ 100 μ l of packed erythrocytes were treated with DIPFP with or without PAM (500 nmoles) for 8 hours. Following treatment, excess reactants were removed and the erythrocytes were lysed by the addition of 0.2% Tween (v/v).

² 500 nmoles of PAM was added at the initiation of the 8 hour incubation.

Discussion

The development of a Western blot assay for the detection of OP-inhibited AChE depends on successful separation of the aged from nonaged enzyme as well as access to an antibody that recognizes an epitope of AChE that is not altered by aging. While we were not able to achieve the separation of AChE required for a useable assay, our experience should be useful to others who are attempting to develop a method that measures AChE protein.

First, we had adequate but limited quantities of antibodies. Despite the limitations, we developed two versions of a Western blot assay to detect AChE. Detection with histochemical stains made it possible to determine the mobility of AChE into the gels, optimize the conditions for transfer to the membrane, and detect the separation of enzyme isozymes. The results were a Western blot using conventional electrophoretic separation and another that used IEF to detect AChE.

Using these methods, several modifications were found to be necessary to achieve complete transfer of the AChE to a membrane for quantitation. Although Immobilon P membrane has a 0.45 μm pore size and is used for most Western blotting applications, a membrane with a smaller pore size of 0.2 μm was required. The retention of proteins on Immobilon P is determined by their structure, hydrophobicity, molecular weight, and transfer conditions. Human erythrocyte AChE is a globular dimer (Massoulie and Bon, 1982) consisting of identical subunits each with molecular weights of 66 kDa (Massoulie et al., 1993; Ott, 1985). Generally, proteins with molecular weights greater than 50 kDa are retained on the membrane. The passage of the enzyme through one and sometimes two backup membranes suggests that the molecule had a highly compact globular structure. Using the 0.2 μm pore PVDF membrane, adequate retention was achieved and the optimal transfer conditions were found to be the highest attainable voltage, usually 55V for 30 minutes.

Initial experiments indicated that inhibited and native AChEs had similar mobilities when subjected to nondenaturing electrophoresis. This was expected to be the case for the higher pHs. However, no separation was apparent when the buffer had an initial pH of 5.8. Although the pH of this buffer exceeds the upper range of the pIs reported for AChE (4.2 to 5.7) (Fritze and Beckmann, 1987), the pH declines as the temperature of the buffer increases. After an hour of electrophoresis, the pH of the buffer was 5.6, within the range of isoelectric points for AChE. Reduced mobility was expected at lower pHs and longer electrophoresis times were anticipated. However, heating of the electrophoresis buffer imposed a limit of three hours at which time no separation was evident. The difficulties of achieving the running time necessary for separation lead us to consider alternative separatory methods.

Isoelectric focusing, capable of separating proteins with isoelectric points differing by as little as 0.02 pH units (Garfin, 1990), was used for subsequent attempts to distinguish inhibited from native AChEs. IEF was found to be a superior method capable of separating the crude AChE preparations into at least 5 major bands having AChE activity.

The resolution and apparent level of activity were improved by substituting Tween 20 for Triton X-100. Both are nonionic detergents which have been previously noted to be necessary for the maintenance of activity in disaggregated human erythrocyte AChE (Wiedmer et al., 1979).

AChE is highly heterogeneous, with the specific forms of AChE determined by the tissue of origin. AChE polymorphisms are not genetic, since all AChEs in vertebrates originate from a single highly conserved gene (Taylor 1992), but are due to alternative splicing and post translational modification (Taylor and Radic, 1994). Erythrocyte AChE includes a globular amphipathic dimer (G_2) which contains the catalytic site (Massoulie and Bon, 1982). In addition to the globular structure, erythrocyte AChE also has a glycoinositol phospholipid tail (Dutta-Choudbury and Rosenberry, 1984; Toutant et al., 1989) which anchors the protein to the membrane. The branched structure of the anchor has been described and the composition of the core oligosaccharides and lipids have been identified (Deeg et al., 1992).

Multiple bands of erythrocyte AChE have been reported (Fritze and Beckmann, 1987; Ott and Brodbeck, 1978) but the molecular structures responsible for the observed heterogeneity have not been identified. These bands could either be true isozymes or AChEs reproducibly degraded by their extraction from the erythrocyte membrane. Separation of the isolated globular portion of the molecule produces two bands, presumably corresponding to the dimer and disassociated monomer (Toutant, 1986). Heterogeneity detectable as multiple bands may be due to fragmentation of the anchor during isolation from the membrane. An additional source of heterogeneity may be attributable to the presence of three glycosylation sites on the surface of the globular portion of the molecule which have been reported to alter electrophoretic mobility (Massoulie et al., 1993).

Despite the ability to resolve AChE variants, none of the bands showed an inhibition-related shift in isoelectric point as evident in Figure 10. While the multiple bands confirmed the superior resolution of IEF compared to electrophoresis, their presence complicated the assessment of the relative mobility of the native and aged AChEs. Only by examining the pattern of bands in multiple lanes was it apparent that no treatment related shift in mobility had occurred. The slight differences in location visible in the Figures showing IEF gels is due to irregularities in the gel evident as ridges. The ridges arise because of discontinuities in the pHs formed by the mixture of ampholytes (personal communication, Pharmacia Technical Support). By following the ridges, it is possible to distinguish bands that have same isoelectric point but are positioned slightly differently due to the gel from bands that have unique isoelectric points. Further evidence that differences in the position of bands were not due to distinct isoelectric points is their failure to correlate with treatment level.

The Western blot assay assumes that the physicochemical difference between the analytes are sufficient to permit separation. Two differences have been reported. The first is the resistance of aged butylcholinesterases to denaturation compared to native enzyme

(Masson et al., 1994). This behavior has not been evaluated in AChE. A distinction that is more amenable to separation by standard analytical methods is the charge difference between native and aged AChE (Segall et al., 1993).

Several scenarios should be considered when evaluating the reasons for the inability to separate native from inhibited enzyme. One explanation is that the model that postulates the acquisition of a negative charge with aging may be incorrect. Until recently, the irreversible inhibition brought about by aging was inferred to be due to phosphorylation at the active site of the enzyme. ^{31}P NMR analysis of the structure of the active site of enzyme inhibited DIPFP, the same OP used for our studies, clearly shows a negative charge in the phosphoryl conjugate that is not present in the native enzyme (Segall et al., 1993). However, the negatively charged phosphoryl conjugate could be detected only after AChE had been denatured with 6M guanidine hydrochloride whereas phosphoryl conjugates of chymotrypsin could be detected in both the intact and denatured molecules (Segall et al., 1993).

Another possibility is the OP-AChEs used in these experiments may not have been aged and therefore the reversibly phosphorylated enzyme carried no additional charge. Treatment conditions and the specific OPs were chosen to favor the production of aged AChEs (Hayes, 1982; Toutant et al., 1989). In addition, the presence of aged AChE was verified by treatment with large molar excesses of PAM. The results showed that irreversible inhibition occurred when at least 300 pmoles of DIPFP or 50 moles of paraoxon were used to treat 100 μl of packed erythrocytes. Other investigators examining the efficacy of paraoxon and DIPFP in producing aging found that while paraoxon produced over 50% inhibition at concentrations of 0.1 μM , only 10% of the AChE was aged (Dehlawi et al., 1994). With DIPFP, concentrations of approximately 1 μM produced 50% inhibition and 40-50% of the AChE was aged (Dehlawi et al., 1994). Although the experimental conditions differed considerably because of immobilization of the AChE, the concentrations are comparable to the concentrations reported in these experiments (600 pmoles/100 μl packed erythrocytes = 0.6 μM).

It is possible that the AChE was phosphorylated but the separatory method was inadequate. This is unlikely since both electrophoresis and IEF are commonly used methods to separate proteins on the basis of phosphorylation and the acquisition of a single charge. In particular, a recent study of *Bacillus subtilis* successfully used an approach virtually identical to the one reported here; IEF was followed by Western blotting to successfully separate transcription factors on the basis of phosphorylation (Arigoni et al., 1996).

Finally, separation may not be feasible because the charge acquired as a consequence of aging does not result in an altered pI. This could be because the quaternary structure of the molecule is such that the charge is internalized. This is consistent with what is known about the quaternary structure of AChE and our observations while developing this assay. In the blot transfer experiments, we found that the behavior of AChE was consistent with a molecule that is compact for its molecular weight suggesting hydrophobicity.

Alternatively, other alterations that accompany aging may also produce changes in the charge such that there is no net gain after the phosphorylation.

The active site of AChE is found in an “aromatic gorge” deep within the molecule (Ripoll et al., 1993). The net charge on the enzyme is -8e due to a nonrandom dispersal of charges around the gorge where negative charges predominate on one side while positive charges predominate on the other side (Ripoll et al., 1993). This nonrandom distribution results in a dipole which guides the substrate into the gorge. The gorge itself contains aromatic residues in a configuration that suggests shielding from the charged molecules on the exterior of the molecule (Ripoll et al., 1993). The presence of an aromatic residue-shielded interior is further supported by the inability to detect charged phosphate conjugate in the intact molecule (Segall et al., 1993).

Based on these observations, the following strategies could be pursued to develop an assay to detect AChE. Methods that disrupt the quaternary structure of the AChE molecule could be used to externalize the phosphate conjugate after aging. These include digestion with papain (Dutta-Choudhury and Rosenberry, 1984), sonication (Massoulie and Bon, 1982), or denaturation by SDS, heating or guanidine hydrochloride. Antibodies capable of detecting epitopes produced by the alteration in enzyme quaternary structure would need to be developed eg. capable of recognizing denatured or fragmented and phosphorylated AChE. The purpose of the papain digestion reported here was to determine whether separation of phosphorylated fragments was possible. This was not successful because both catalytic activity and the antibody epitope were lost during the digestion.

Alternatively, rather than separating the native from aged AChE, antibodies that detect phosphorylated protein could be developed. These could be combined with antibodies such as those used in this investigation, that do not distinguish between aged and native AChE. A drawback of this approach comes from the nature of the phosphorylation and adduction of the AChE which depends on the structure of the parent OP. As a result, immunoassays of this type would detect specific OPs rather than the response to an entire class of compounds, as is the case with measures based in inhibition of catalytic activity.

Finally, a simple approach that would require only optimization of existing assays rather than developing new methods would be to express catalytic activity per unit of protein (pmol/min/mg AChE). Since this approach uses two different assays, one for AChE activity and another for quantitation of the protein, the accumulated error would probably make it difficult to identify very levels of inhibition below ~10%. However, it would be possible to measure OP-induced inhibition in the absence of pre-exposure screening in instances where it is not currently possible.

Conclusions

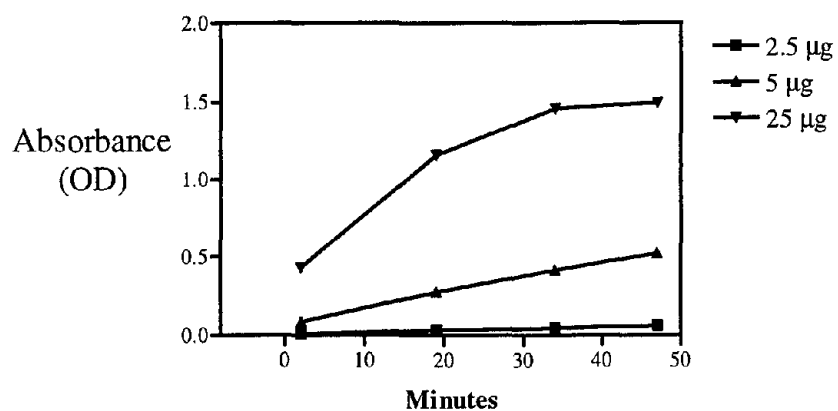
Refer to Table 1 which lists the specific aims:

- I. The mobility of AChE after nondenaturing PAGE electrophoresis was verified with a histochemical stain.
- II. The transfer of AChE from the PAGE membrane was accomplished using high voltage (up to 100V), short duration transfers (30 minutes) and a 0.2 μ m membrane.
- III. Qualitative experiments to determine whether enzyme inhibition influences the recognition by the HR antibodies showed no treatment-related pattern of recognition
- IV. Immunochemical detection methods were optimized on the Western blotted membrane rather than pursuing a separate Western dot blot.
- V. Several strategies were used to accomplish the separation of native and inhibited/aged enzyme in the IN assay. Electrophoresis was performed using buffers near the isoelectric point of AChE. When isoelectric focusing was substituted for the electrophoresis, at least 5 isozymes of AChE could be resolved. However, treatment-related shifts in pI were not observed in any of the isozymes when using AChE in which aging had been confirmed.
- VI. Experiments aimed at separating the inhibited/aged from native AChE were performed using DIPFP and paraoxon.

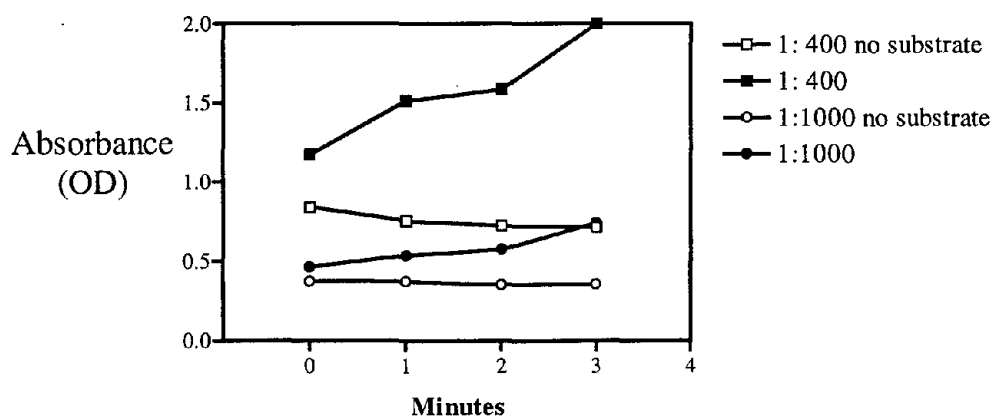
In conclusion, the method found to be most effective at separating AChEs was IEF rather than electrophoresis as evidenced by the ability to resolve at least 5 variants of AChE. Using this approach, we attempted separation of DIPFP- and paraoxon-inhibited AChE which our studies with PAM, an agent which reverses inhibition in non-aged enzyme, showed to be predominantly aged. No treatment-related differences in pI were identified in any of the AChE variants. The inability to separate the inhibited/aged from native enzyme precluded further quantitative studies to determine detection limits, compare the results/variability obtained with the IN and Ellman's assays, or detect exposure. We conclude that alternative approaches to distinguishing aged from native AChE should be pursued since it is probable that the architecture of the active site within the hollow center of the AChE molecule shields the charge conferred by the phosphorylation and prevents separation.

Planned Publications

This report will be revised into a manuscript for publication pending additional experiments repeating the papain digestion and quantitative assessment of activity after Western blotting. Results will be expressed as AChE activity per unit of AChE protein.



A. commercial human AChE



B. erythrocyte lysate

Figure 1. AChE activity measured colorimetrically with Ellman's reagent. (A) commercial crude preparation, µg total protein. (B) dilutions of erythrocyte lysate made from 100 µl packed erythrocytes in a final volume of 1 ml.

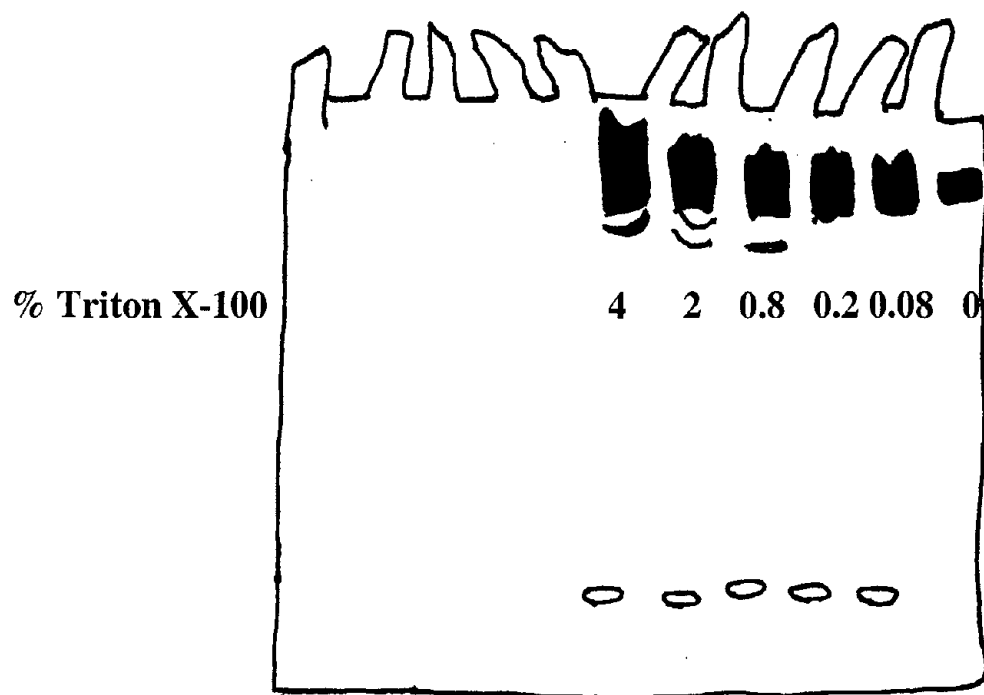
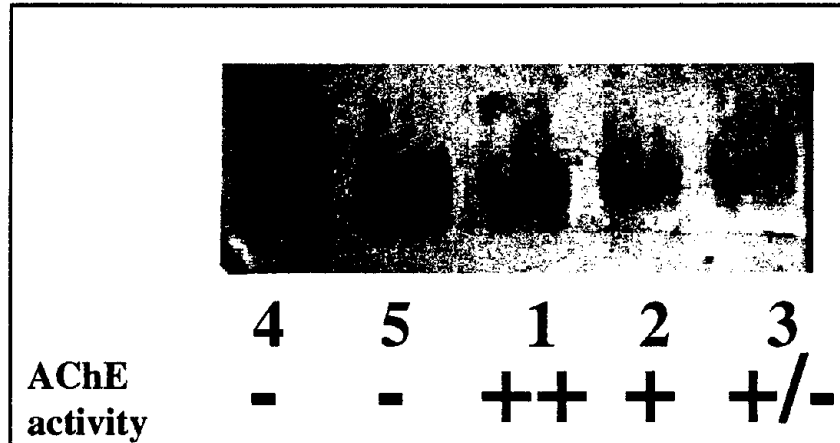
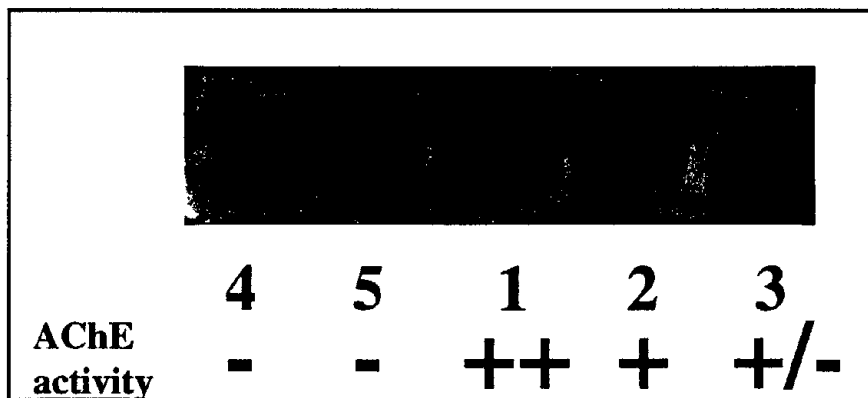


Figure 2. AChE activity in erythrocytes lysed with different percentages of Triton X-100. Separation by nondenaturing electrophoresis as described in the Materials and Methods using 5 μ l of lysate in each well. .



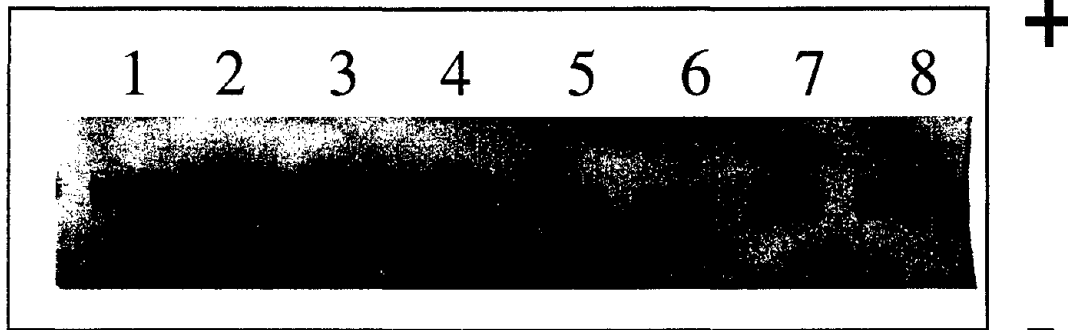
A. HR2



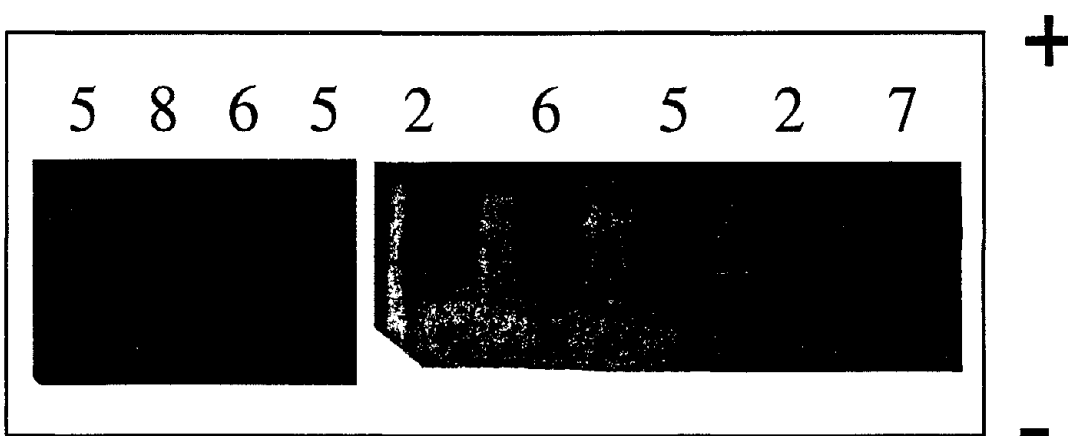
B. HR3

Figure 3. HR antibody recognition of native and DIPFP-inhibited AChE after electrophoresis. Erythrocytes were treated with DIPFP as follows: (lane 1) untreated; (lane 2) 900 pmoles; (lane 3) 1500 pmoles; (lane 4) 8700 pmoles; (lane 5) 28,800 pmoles

Each reaction used 100 μ l of packed erythrocytes in 900 μ l 0.85% NaCl & 15 mM NaH_2PO_4 . After 2 hours, erythrocytes were washed followed by lysis with H_2O and 0.2% Tween. Electrophoresis was performed with 5 μ l of lysate in each well.



A. electrophoresis



B. isoelectric focusing

Figure 4. Relative mobilities of paraoxon-treated AChE detected by Western blot using separation by either electrophoresis (A) or isoelectric focusing (B). Lysate from untreated erythrocytes was mixed with lysate containing AChE inactivated by treating 100 μ l of packed erythrocytes with 5 μ moles of paraoxon. The proportion of inactivated lysate is as follows: (lane 1) 75%; (lane 2) 50%; (lane 3) 25%; (lane 4) 10%; (lane 5) 0%; (lane 6) 100%; (lane 7) treated lysate incubated with 500 nmoles PAM; (lane 8) lysate from 100 μ l erythrocytes treated with 50 μ moles of paraoxon. Each lane contains equivalent quantities of lysate.

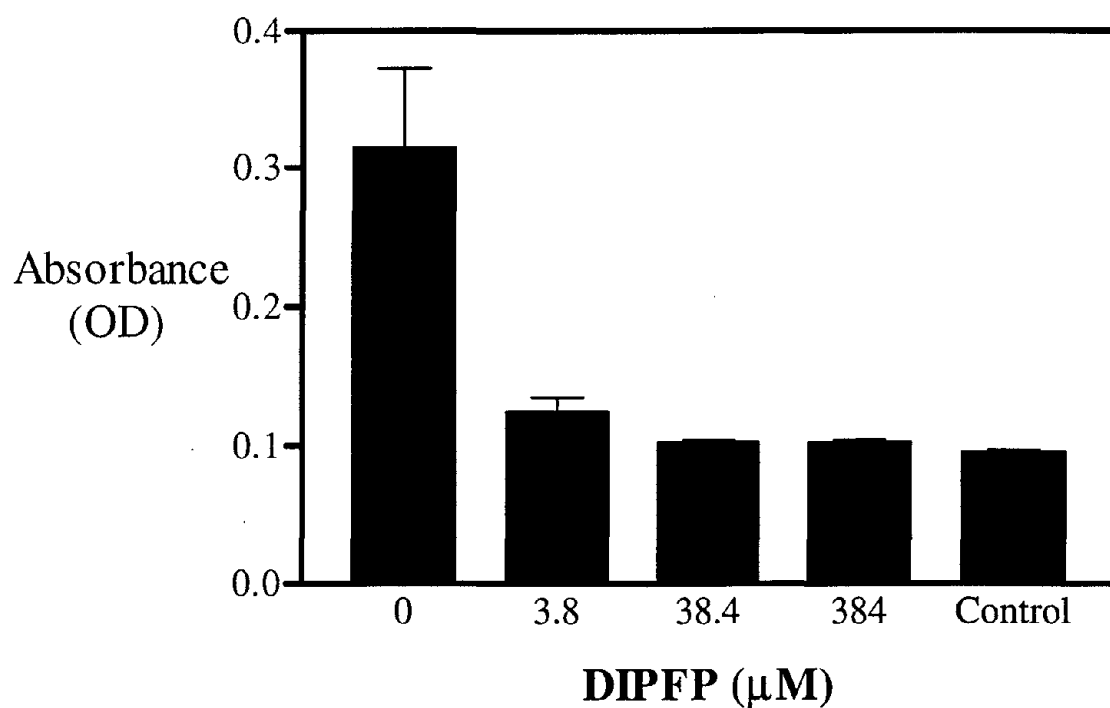


Figure 5. AChE activity in erythrocyte ghosts treated with DIPFP. Activity measured colorimetrically using Ellman's reagent according to the Cal EPA method (1994). Each reaction used ghosts equivalent to 75 μ l packed erythrocytes. The control reaction was done in the absence of AChE but contained all other reagents.

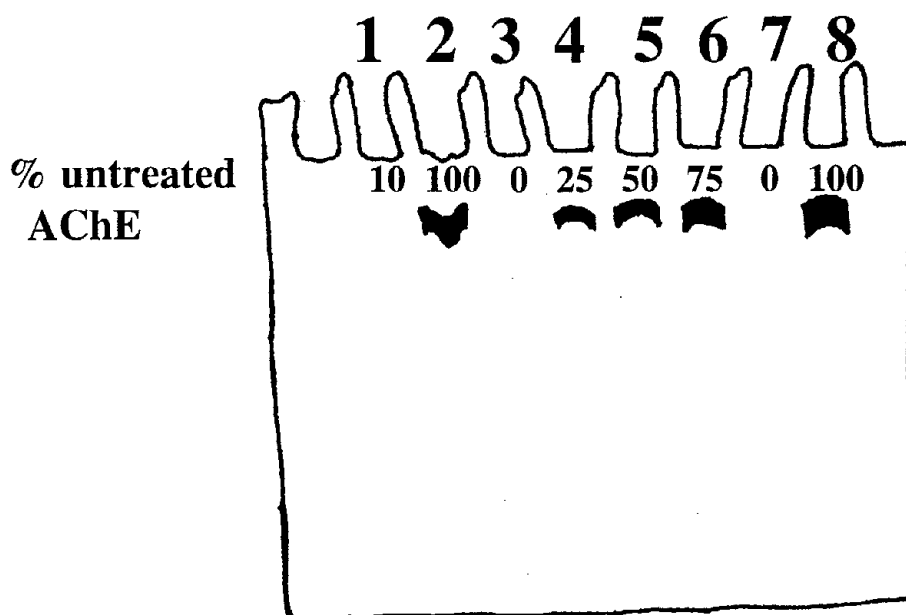


Figure 6. AChE activity detected in erythrocyte lysates containing different proportions of DIPFP-treated AChE. Each numbered lane contains equivalent quantities of AChE. The intensity of the staining corresponds to the proportion of untreated, hence active AChE. Detection of activity was according to the method of Karnovsky and Roots (1964). Separation by electrophoresis using 5 μ l of lysate in each well.

AChE was inactivated by incubating 100 μ l packed erythrocytes with 300 pmoles of DIPFP for 2 hours. The total volume was maintained at 1 ml throughout. The erythrocytes were washed to remove excess DIPFP followed by lysis with H₂O and Triton X-100.

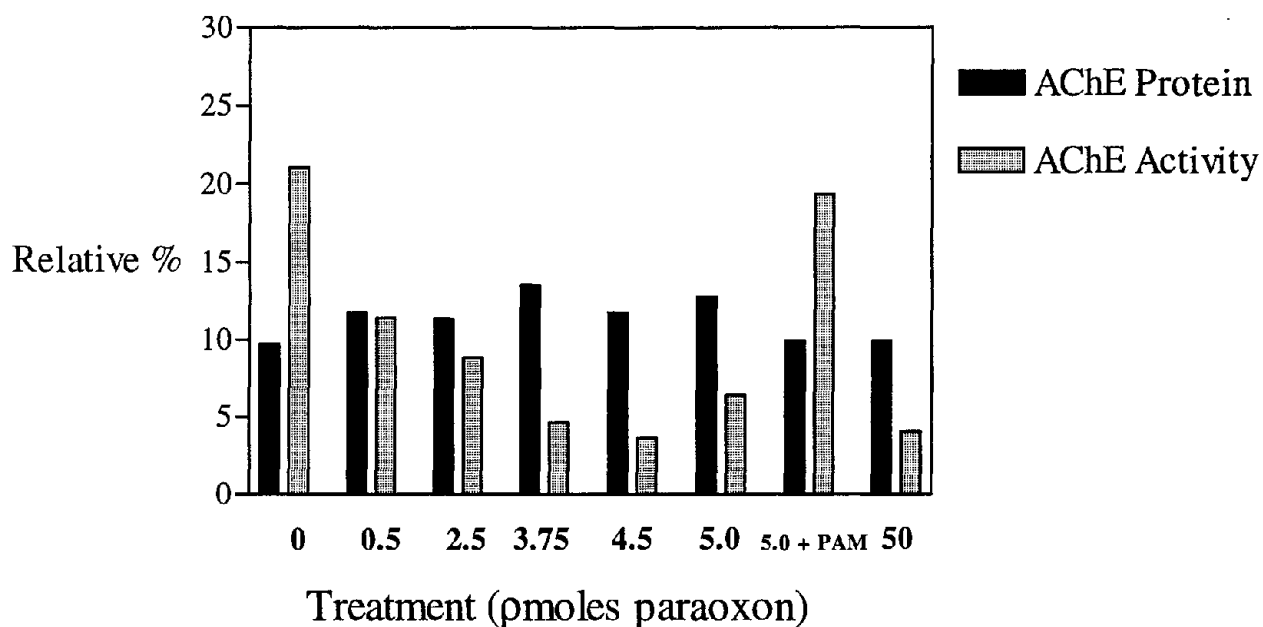


Figure 7. Detection of AChE protein and catalytic activity after treatment with paraoxon. Each treatment level used 100 μ l of packed erythrocytes which were incubated with paraoxon for 2 hours, then washed and lysed with 0.2% Tween. Electrophoresis was performed with 5 μ l of lysate per well. AChE protein was detected by Western blot using anti-AChE HR2. AChE activity was assayed according to the method of Karnovsky and Roots (1964).

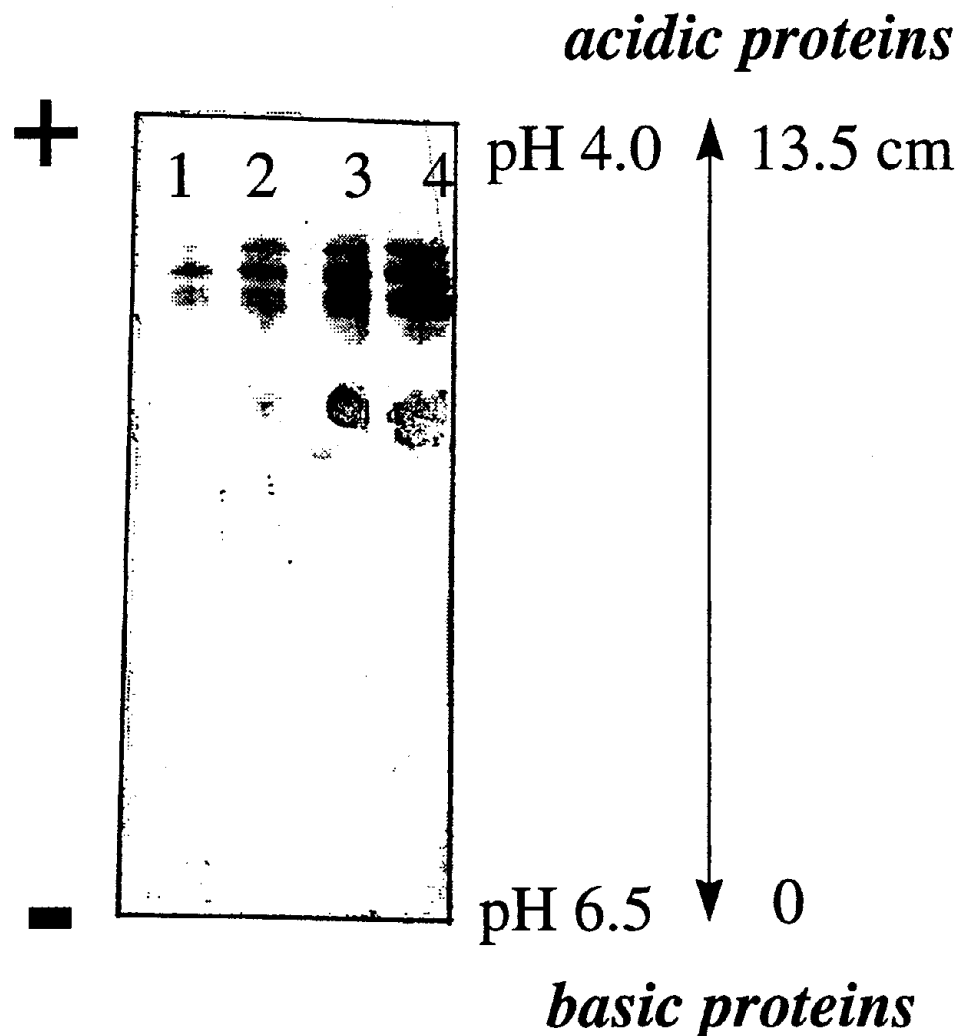


Figure 8. AChE activity on a PAGE gel after separation by isoelectric focusing. Lysate was prepared from 100 μ l of packed erythrocytes by the addition of 0.2% Triton X-100. Lysate volumes used for isoelectric focusing were: (lane 1) 0.10 μ l; (lane 2) 0.25 μ l; (lane 3) 1 μ l; (lane 4) 2 μ l.

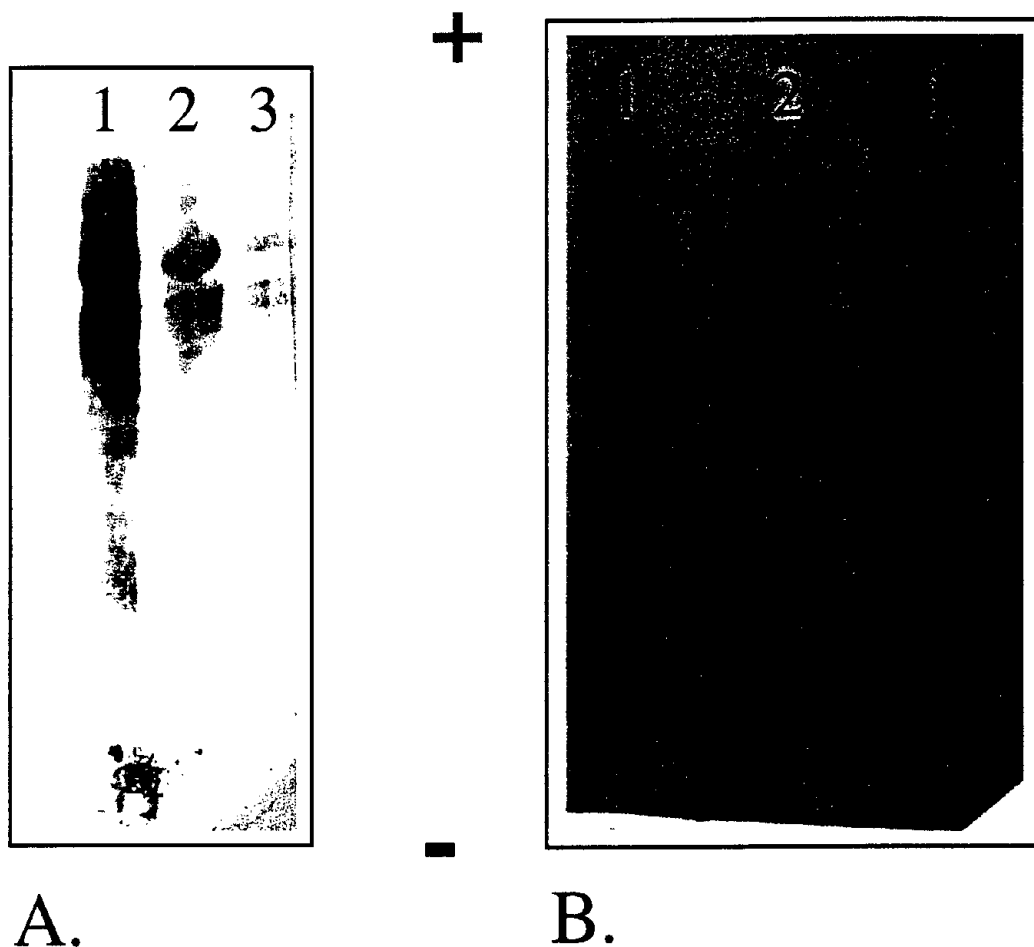


Figure 9. Detection of AChE activity (A) and AChE protein (B) after isoelectric focusing. 100 μ l of packed erythrocytes were treated with DIPFP as follows: (lane 1) untreated; (lane 2) 3600 pmoles; (lane 3) 9000 pmoles.

Erythrocytes were maintained in 1 ml during 2 hour incubation with DIPFP, then lysed with 0.2% Tween. IEF used 3 μ l of lysate per lane. Detection of activity in (A) was done according to the method of Karnovsky and Roots (1964) using a thiocholine substrate. In (B), immunochemical detection used murine anti-AChE (HR2; 1:2500), NBT/BCIP substrate.

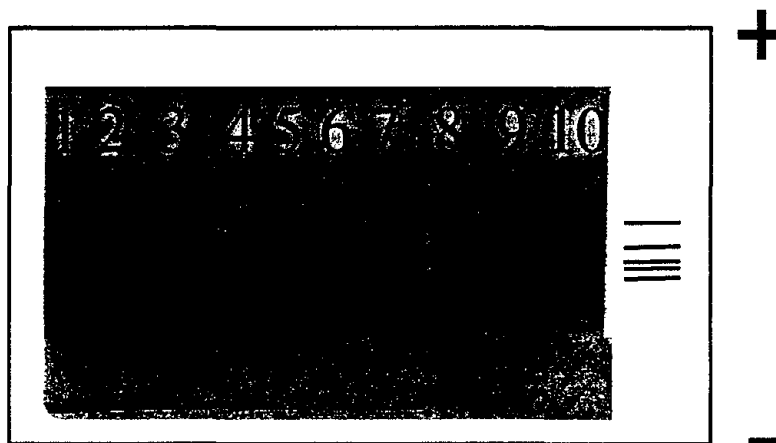


Figure 10. Mobilities of native and DIPFP-treated AChE compared by Western blotting using separation by IEF. DIPFP treatment per 100 μ l packed erythrocytes: (lanes 1 & 7) 600 μ moles; (lanes 2, 6, 8) 50% 5800 μ moles & 50% untreated; (lanes 3, 5, 10) untreated; (lanes 4, 9) 5800 μ moles

Erythrocytes were maintained in 1 ml 0.85% NaCl and 0.15 mM Na_2HPO_4 during treatment with DIPFP for 2 hours. Following treatment, erythrocytes were washed three times, then lysed with 0.2% Tween. Each lane contains 1 μ l of lysate. Immunochemical detection used murine anti-AChE (HR2; 1:2000) and NBT/BCIP substrate.

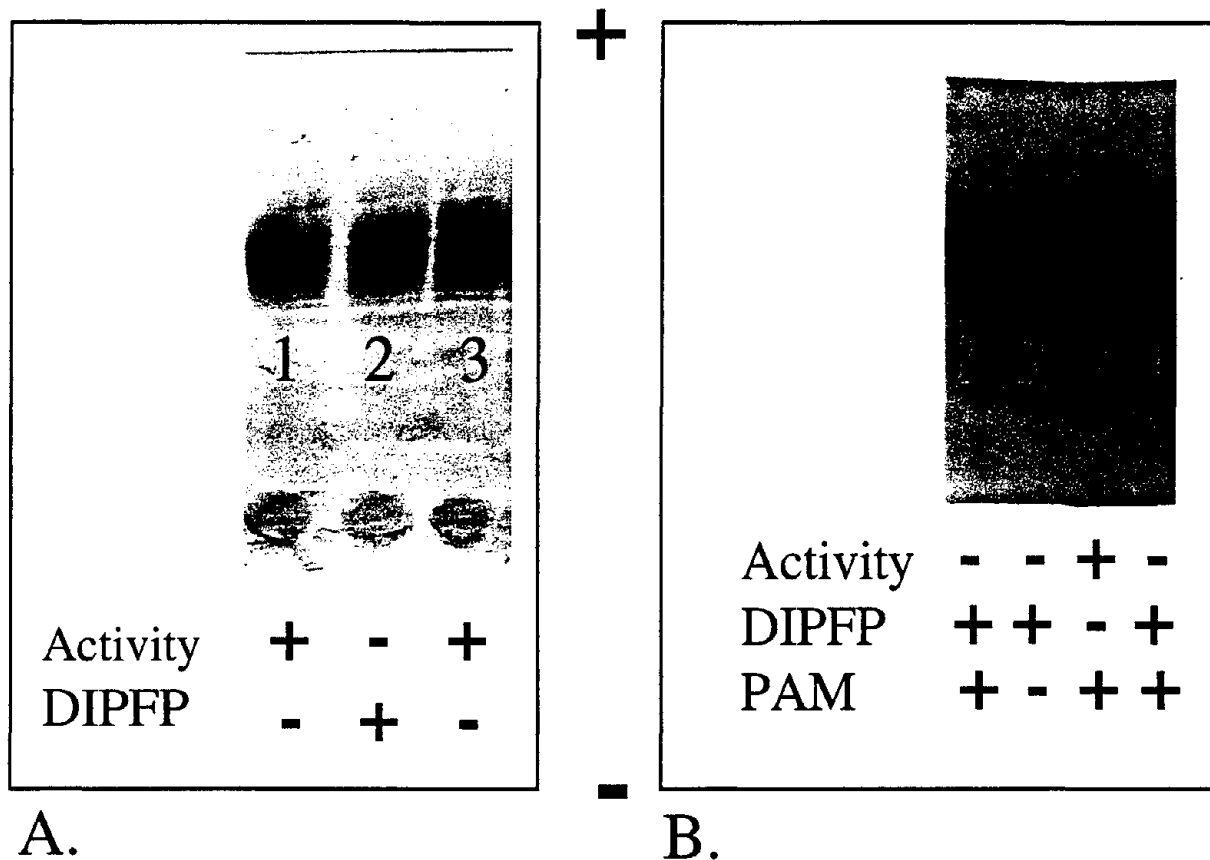


Figure 11. Relative mobilities of native, DIPFP- and PAM-treated AChE separated by IEF and detected by Western blot. (A) (lanes 1 & 3) untreated control lysate; (lane 2) 100 μ l packed erythrocytes treated with 300 μ moles DIPFP; (B) (lanes 1 & 4) 100 μ l packed erythrocytes treated with 600 μ moles DIPFP, and 150 nmoles PAM; (lane 2) DIPFP only; (lane 3) untreated control.

Erythrocytes were maintained in 1 ml during the 8 hour incubation with DIPFP/ PAM, then washed three times and lysed with 0.2% Tween. Each lane used 2 μ l of lysate. Immunochemical detection with murine anti-AChE (HR2; 1:2000), NBT/BCIP substrate.

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