THE UNIVERSITY OF MANITOBA

STUDIES ON ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE

BY

EVELYN M. KINSCH

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DEDICATION

To my father, and in loving memory of my mother.

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I would like to express my deepest and most heartfelt thanks to my supervisor, Dr. George Tomlinson for his continued support, encouragement and guidance throughout this project. I would also like to thank Dr. Oksana Lockridge, Dr. Bert LaDu and Dr. Larry Schaefer for the assistance, kindness, and hospitality shown to me during my stay in Ann Arbor, Michigan.

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Finally, I would like to express my sincere thanks to my husband Neil, for his love, encouragement, and mostly for being there.

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ABSTRACT

The work in this thesis will be presented in two parts. Firstly, an evaluation of S-mercuric-N-dansylcysteine as a potential probe for the the detection of free SH groups in *Torpedo californica* acetylcholinesterase (AChE) and Human serum butyrylcholinesterase (BChE); and the effects of metal ions on the spectral and kinetic properties of the labeled enzymes. Secondly, a short study of the interaction of *Elecrophorus electricus* AChE with arsenocholine and acetylarsenocholine.

S-mercuric-N-dansylcysteine (SMNDC) was investigated as a potential probe of protein sulfhydryl groups using bovine serum albumin (BSA), S-carboxymethyl-BSA, lysozyme and partially reduced lysozyme as model proteins. A set of criteria was developed for assessment of covalent binding of the SMNDC to proteins through a mercaptide linkage as follows: a finite reaction time, abolition of the characteristic fluorescence spectrum following addition of a reducing agent, and failure to separate probe and protein after gel filtration chromatography or SDS-polyacrylamide gel electrophoresis. Examination of the interaction of the probe and *T. californica* AChE, *E. electricus* AChE, and human serum BChE, revealed that both *T. californica* AChE and BChE contain four free sulfhydryl groups per tetrameric enzyme molecule whereas *E. electricus* AChE has none. Labeled AChE and BChE remain active and responsive to Zn^{2+}, an inactivator of both enzymes. Zn^{2+} promotes an increase in bound SMNDC fluorescence whereas activators such as $Mg^{2}{}^{2+}$ or gallamine promote a decrease, suggesting that SMNDC may be a useful probe of ligand-induced conformational changes. With *T. californica* AChE only, Zn^{2+} promotes access to two additional SMNDC-

reactive groups.

In the second part of this study, the properties of arsenocholine as an inhibitor of AChE and acetylarsenocholine as both substrate and inhibitor were examined. Both compounds were found to be moderately potent competitive inhibitors of acetylthiocholine hydrolysis by *E. electricus* AChE, with K_i values 96.2 μM and 42.3 μM, respectively. Acetylarsenocholine was also shown to be a substrate of AChE, with similar properties to that of the natural substrate acetylcholine. Thus, K_m for acetylarsenocholine was 482 μM (compared to 408 μM for acetylcholine) and the relative maximum velocity of hydrolysis (acetylcholine/acetylarsenocholine) was 1.14. Preliminary data indicated that the inhibition of acetylcholine hydrolysis by arsenocholine contained a marked uncompetitive component; however, the low precision of the data prevented any firm conclusions from being drawn.

LIST OF ABBREVIATIONS

ACh Acetylcholine

AcArCh Acetylarsenocholine

AChE Acetylcholinesterase (E.C.3.1.1.7)

ArCh Arsenocholine

ASCh Acetylthiocholine

BChE Butyrylcholinesterase (serum cholinesterase, E.C. 3.1.1.8)

BSA Bovine serum albumin

Cbz Carbobenzoxy

DMB Decamethonium bromide

DTNB 5,5'-Dithiobis-(2-nitrobenzoic acid)

DTT Dithiothreitol

EDAC 1-ethyl-3-(3-dimethylpropyl)-carbodiimide

EQB Equilibration buffer

M7C N-Methyl-7-(dimethylcarbamoyloxy) quinolinium iodide

M7H N-Methyl-7-hydroxyquinolinium iodide

PTA Phenyltrimethylammonium

SDS Sodium dodecyl sulfate

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMNDC S-mercuric-N- dansylcysteine

TEMED N,N,N',N'-tetramethylethylenediamine

TPB Torpedo preparation buffer

PART I

AN INVESTIGATION OF THE THIOL GROUPS IN ACETYLCHOLINESTERASE AND HUMAN SERUM CHOLINESTERASE

INTRODUCTION

i) Historical background

The concept of chemically-mediated neurotransmission was first introduced in 1904 by T. R. Elliot, who suggested that adrenalin may be a chemical transmitter from the sympathetic nerves to their effector organs (1). This was based on the observation that an extract of adrenal gland (adrenalin) could mimic the actions of sympathetic nerves. The notion of chemical transmission was not well received because of the entrenched belief that communication between cells, like the nerve impulse itself, was also electrical in nature (2). Over forty years were to pass before it was finally accepted that noradrenaline (rather than adrenalin) was a chemical mediator of sympathetic neurotransmission (3).

The establishment of the neurotransmitter role of acetylcholine required a similarly protracted period. The pharmacological actions of this substance were first described in 1906 by Hunt and Traveau (4). From their work, and that of many others, it was suggested that acetylcholine was released from certain nerve endings and crossed the nonconducting gap to act as an intercellular chemical mediator of nerve impulses. The release and removal of acetylcholine were discovered to be processes which occurred in the active membrane surrounding the nerve fibre (5). Cole and Curtis (6) confirmed that acetylcholine produced a change in the permeability of the nerve cell membrane, allowing acetylcholine to pass through and act as a trigger for the

excitation of the adjacent nerve or muscle cell. The hydrolysis of acetylcholine into its inactive components, acetic acid and choline, restored the resting condition of the membrane.

The results of pharmacological experiments led Dale in 1914 to propose the existence of an enzyme capable of catalyzing the hydrolysis of acetylcholine to acetic acid and choline in nervous tissue (7). A cholinesterase was found in horse serum by Stedman et al. in 1932 (8) but was subsequently shown to be not specific for acetylcholine (9). It was later observed by Alles and Hawes in 1940 (10), that there were two types of cholinesterase in blood: one associated with red cells and one for serum. They were differentiated by the fact that the red cell esterase had a well defined optimum substrate concentration (in that excess substrate caused a sharp decrease in activity) in contrast to the serum esterase which followed typical Michaelis-Menten kinetics and did not display substrate inhibition. Nachmansohn was the first to demonstrate the presence of a cholinesterase in nerve and muscle tissues (11), the enzyme activity being associated with particulate fractions of these tissues (12). On the basis of these different substrate specificities, and their different sensitivities towards a variety of inhibitors, the cholinesterases were divided into two classes by Augustinsson and Nachmansohn (13): the true cholinesterase was named acetylcholinesterase (acetylcholine acetylhydrolase, E.C. 3.1.1.7, found in erythrocytes, nerve and muscle), and the others were named pseudo-, serum, or butyryl-choline esterase (acylcholine acylhydrolase, E.C. 3.1.1.8). These are hereafter designated as AChE and BChE, respectively.

In order to be classified as an acetylcholinesterase the enzyme

must have a high affinity for acetylcholine, show substrate inhibition and a substrate preference whereby the rate of hydrolysis declines with respect to increasing length of the acyl chain (acetylcholine > propionylcholine > butyrylcholine). Serum cholinesterases (BChE) are classified according to their high affinity for butyrylcholine. There is no substrate inhibition and the rate of substrate hydrolysis increases in the order acetylcholine < propionylcholine < butyrylcholine (12-15).

Acetylcholinesterase has been found in a wide variety of tissues such as invertebrate and vertebrate motor, sensory, central and peripheral nerve, (both cholinergic and adrenergic), and in striated muscle. Varying amounts are found in all tissues; however, the highest concentrations are found in places which require high levels of acetylcholine hydrolysis during very short periods of time. The discovery of increased concentrations of AChE in muscle, especially at the endplate regions (16), led Nachmansohn to examine the activity of the electric organs of the electric eel and Torpedo ray (17,18), since these are essentially modified neuromuscular junctions. The availability of relatively enormous quantities of enzyme from these sources has made possible a wide range of chemical, structural, kinetic and pharmacological studies spanning the past five decades.

ii) Molecular forms of acetylcholinesterase

Early methods of isolation of solubilized (11s) tetrameric AChE from *Electrophorus electricus* electroplax employed autolysis of tissue under toluene for several months (19). Subsequently, it was found that

limited proteolysis using trypsin accomplished much the same results in a far shorter time (20). The main implications of these studies were clearly that soluble AChE was derived by proteolysis from a more complex structure.

More recent studies have shown that native AChE exists in a variety of molecular forms (128). Both globular and asymmetric, 'tailed', species were found in *E. electricus* (21,22) and *T. californica* (23) electroplax. It is now generally understood that the cholinesterases are polymorphic enzymes which fall into two broad groups. According to the terminology introduced by Bon (24), these are referred to respectively as Globular (G) and Asymmetric (A). Within each group are several members having characteristic molecular weights, sedimentation coefficients, and subunit compositions.

The asymmetric forms all contain a collagen-like, filamentous 'tail' unit which is disulphide-linked to one (A_4), two (A_8) or three (A_{12}) tetrameric sets of catalytic subunits. The tetramers consist of four identical 70kDa polypeptide chains, of which two are disulphide-linked to form a dimer which noncovalently associates with the other two tail-associated subunits (25,26). These high molecular weight forms of AChE, while soluble in high ionic strength media, form insoluble aggregates at low ionic strength. Limited proteolysis by either trypsin or collagenase cleaves most of the 'tail' material and liberates the soluble (11s) form of the enzyme which has been employed in most studies of AChE function to date. *Torpedo californica* AChE has been reported to contain an additional, noncollagenous, 100kDa subunit of unknown function (27). The role of the collagen-like 'tail' is thought to be in locating the enzyme at the neuromuscular junction by

noncovalent interaction with the basal lamina (23, 27).

The globular forms show considerable variety with respect to both subunit assembly (monomers to tetramers) and hydrophobicity, ranging from totally soluble species to highly hydrophobic forms containing specific glycophospholipids attached to the C-terminal carboxyl group. These membrane-bound enzymes are widely-distributed throughout the nervous system, as well as being found in the membranes of erythrocytes (27, 28,29). The G_4 form is the predominant form of butyrylcholinesterase found on blood serum. It, too, consists of a dimer of disulphide-linked dimers, all four polypeptide chains being identical (30-35).

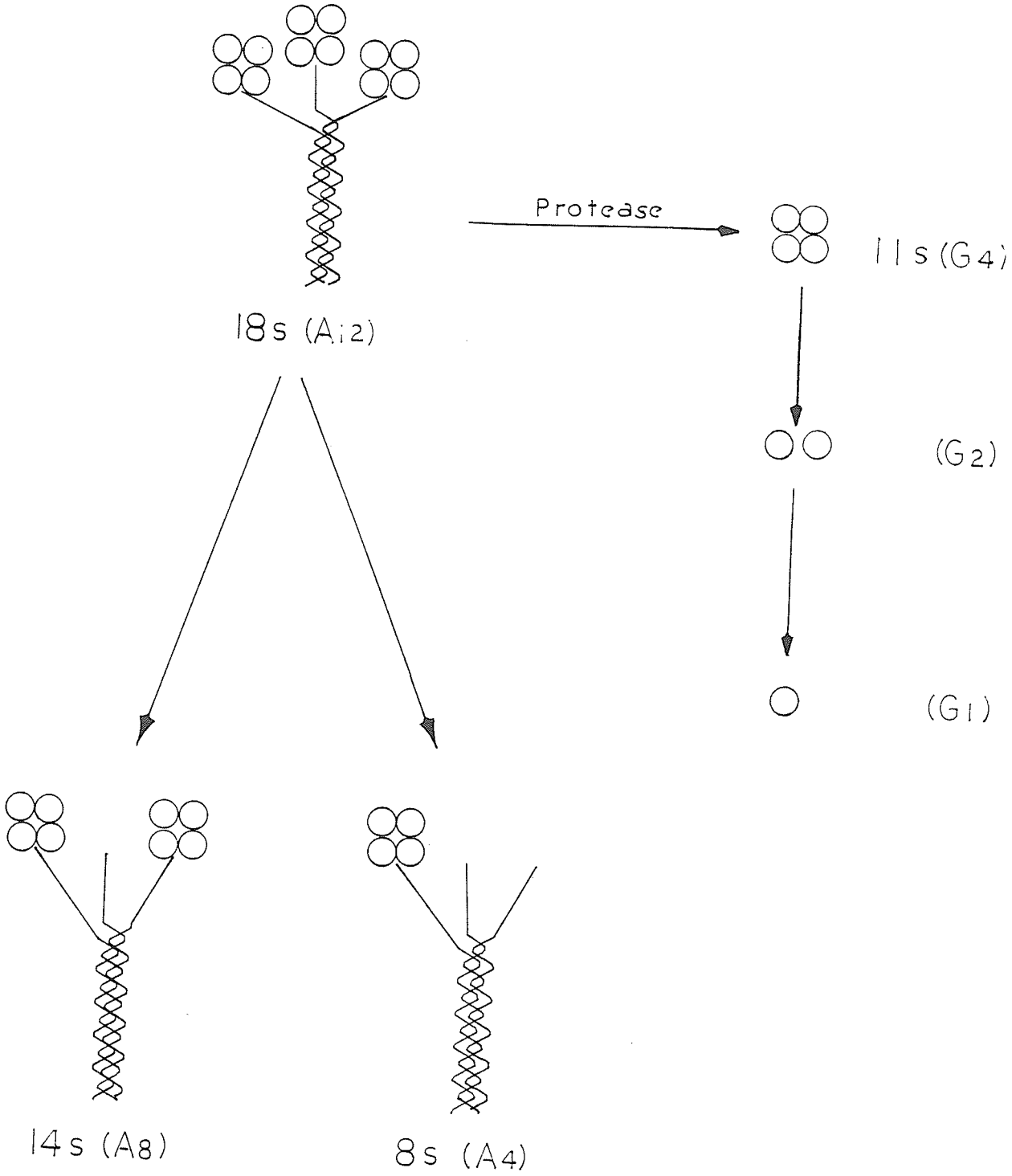
Fig. 1 illustrates some relationships between the various asymmetric and globular forms of the enzyme.

iii) Primary structure of acetylcholinesterase

The primary structures of *T. californica* AChE and Human serum cholinesterase have been recently determined using both conventional protein-sequencing methods and cDNA clone sequencing (36-38). The amino acid sequence for *T. californica* AChE deduced by Schumacher *et al.* (36) shows that the native enzyme contains a single polypeptide of 575 amino acid residues. There are three disulfide loops at cys^{67}-cys^{94}, cys^{254}-cys^{265}, and cys^{402}-cys^{521}, an intersubunit disulfide involving cys^{572}, and a free sulfhydryl group at cys^{231} (39). The amino acid sequence of BChE as determined by Lockridge and her co-workers (37), also shows a single polypeptide of 574 amino acids (one less than in *Torpedo* AChE) with intrasubunit disulfide bridges at cys^{66}-cys^{92}, cys^{252}-cys^{263}, cys^{400}-cys^{519}, an intersubunit

Figure 1. Asymmetric (A) and globular(G) forms of acetylcholinesterase.

All asymmetric forms are derived from the 18S (A_{12}) enzyme, which contains 12 catalytic subunits arranged in three sets of tetramers attached covalently to a collagen-like tail. The 14S (A_8), and 8S (A_4) species represent different levels of subunit assembly. Proteolytic cleavage of the collagen tail results in the formation of the soluble, globular 11S (G_4) form. This can be further dissociated into the G_2 (dimer) and G_1 (monomer) forms. All species are catalytically active.


Protease
18s (A12)
11s (G4)
(G2)
(G1)
14s (A8)
8s (A4)

disulfide at cys^{571}, and a potentially free sulfhydryl group at cys^{66}. Despite their differences in substrate specificities and biological origins, the two enzymes exhibit a 54% sequence homology which, in conjunction with the similar locations of the disulphide bridges, suggests that the two enzymes likely have similar tertiary structures. Differences in subunit molecular weight (70kDa for AChE *vs* 80kDa for BChE) are attributable to the degree of glycosylation, with AChE having four carbohydrate chains per subunit and BChE having nine. The sequences of AChE and BChE are shown in Figs. 2 and 3, respectively.

As might be expected, the peptide sequences in the neighbourhood of the catalytically important serine residue (Ser^{200} in AChE and Ser^{198} in BChE) are highly conserved and the same hexapeptide sequence (Phe-Gly-Glu-SER-Ala-Gly) appears not only in *T. californica* AChE and human serum BChE, but also in AChE from *T. marmorata* (40) and *Drosophila* (41), as well as horse serum BChE (42). Although the cholinesterases appear to have catalytic properties quite analogous to those of the serine proteases, which function through the action of a so-called catalytic triad (Asp, His, Ser (43); see below), the location of the crucial Asp and His residues is not known for either AChE or BChE. It should be stressed that the cholinesterases bear no significant homologies with the serine proteases and appear to comprise an evolutionary distinct family of serine esterases (44).

iv) Catalytic properties

The hydrolysis of acetylcholine to acetic acid and choline by AChE occurs on the region of protein surface known as the active or

Figure 2. Primary structure of *T. californica* AChE, as deduced from its c-DNA clone sequence (36).

```
                    16                  31                  46
G AAT TCC GTG CTT CTG CAC TTG GTC GTC CTG TGC CAG GCG GAC GAT CAC TCT
  Asn Ser Val Leu Leu His Leu Val Val Leu Cys Gln Ala Asp Asp His Ser
                                                    1
        61                  76                  91                 106
GAG CTC CTG GTC AAC ACC AAG TCG GGA AAA GTC ATG GGA AGA AGA GTC CCA GTC
Glu Leu Leu Val Asn Thr Lys Ser Gly Lys Val Met Gly Thr Arg Val Pro Val
  5
               121                 136                 151
CTC TCC AGC CAC ATC AGC GCT TTC CTG GGG ATT CCC TTT GCC GAG CCT CCA GTT
Leu Ser Ser His Ile Ser Ala Phe Leu Gly Ile Pro Phe Ala Glu Pro Pro Val
 33
       166                 181                 196                 211
GGG AAC ATG AGG TTC AGG AGA CCT GAG CCC AAG AAA CCG TGG TCG GGA GTC TGG
Gly Asn Met Arg Phe Arg Arg Pro Glu Pro Lys Lys Pro Trp Ser Gly Val Trp
 41
               226                 241                 256
AAC GCT TCC ACC TAT CCC AAC AAC TGC CAG CAG TAC GTT GAC GAG CAG TTC CCT
Asn Ala Ser Thr Tyr Pro Asn Asn Cys Gln Gln Tyr Val Asp Glu Gln Phe Pro
 59
271                 286                 301                 316
GGA TTT TCA GGT TCA GAG ATG TGG AAT CCG AAC AGA GAG ATG AGT GAG GAC TGT
Gly Phe Ser Gly Ser Glu Met Trp Asn Pro Asn Arg Glu Met Ser Glu Asp Cys
 77
       331                 346                 361                 376
TTG TAC CTC AAC ATT TGG GTG CCT TCT CCG AGG CCG AAG AGT ACA ACC GTC ATG
Leu Tyr Leu Asn Ile Trp Val Pro Ser Pro Arg Pro Lys Ser Thr Thr Val Met
 95
               391                 406                 421
GTG TGG ATC TAC GGA GGC GGT TTC TAC AGC GGG TCC TCG ACG TTG GAC GTC TAC
Val Trp Ile Tyr Gly Gly Gly Phe Tyr Ser Gly Ser Ser Thr Leu Asp Val Tyr
113
       436                 451                 466                 481
AAT GGG AAA TAC CTT GCC TAC ACC GAG GAG GTG GTG CTG GTC TCT CTG AGC TAC
Asn Gly Lys Tyr Leu Ala Tyr Thr Glu Glu Val Val Leu Val Ser Leu Ser Tyr
131
               496                 511                 526
CGG GTG GGC GCT TTT GGT TTT CTC GCC CTC CAC GGC AGC CAG GAG GCA CCA GGA
Arg Val Gly Ala Phe Gly Phe Leu Ala Leu His Gly Ser Gln Glu Ala Pro Gly
149
541                 556                 571                 586
AAT GTG GGC CTC CTG GAC CAG AGG ATG GCA CTG CAG TGG GTG CAC GAC AAC ATC
Asn Val Gly Leu Leu Asp Gln Arg Met Ala Leu Gln Trp Val His Asp Asn Ile
167
       601                 616                 631                 646
CAG TTC TTC GGC GGG GAC CCC AAG ACG GTG ACC ATC TTC GGA GAG AGT GCC GGC
Gln Phe Phe Gly Gly Asp Pro Lys Thr Val Thr Ile Phe Gly Glu Ser Ala Gly
185
               661                 676                 691
GGC GCC TCT GTC GGC ATG CAC ATT CTC TCC CCG GGG AGC CGA GAC CTC TTC CGC
Gly Ala Ser Val Gly Met His Ile Leu Ser Pro Gly Ser Arg Asp Leu Phe Arg
203
    706                 721                 736                 751
CGG GCC ATC CTT CAG AGC GGC TCG CCC AAT TGC CCG TGG GCG TCT GTC TCT GTT
Arg Ala Ile Leu Gln Ser Gly Ser Pro Asn Cys Pro Trp Ala Ser Val Ser Val
221
            766                 781                 796
GCT GAA GGC CGC AGG AGG GCG GTC GAG CTG GGA AGA AAC CTC AAC TGT AAC CTC
Ala Glu Gly Arg Arg Arg Ala Val Glu Leu Gly Arg Asn Leu Asn Cys Asn Leu
239
811                 826                 841                 856
AAC AGC GAC GAA GAG CTC ATC CAC TGT CTG AGG GAA AAG AAG CCT CAG GAG TTG
Asn Ser Asp Glu Glu Leu Ile His Cys Leu Arg Glu Lys Lys Pro Gln Glu Leu
257
       871                 886                 901                 916
ATT GAC GTG GAG TGG AAT GTC CTT CCC TTT GAC AGT ATC TTC AGG TTC TCC TTC
Ile Asp Val Glu Trp Asn Val Leu Pro Phe Asp Ser Ile Phe Arg Phe Ser Phe
275
               931                 946                 961
GTT CCC GTC ATC GAT GGG GAA TTC TTC CCA ACC TCC CTG GAA TCT ATG TTG AAC
Val Pro Val Ile Asp Gly Glu Phe Phe Pro Thr Ser Leu Glu Ser Met Leu Asn
293
    976                 991                1006                1021
TCT GGC AAC TTC AAG AAG ACT CAG ATC TTA CTG GGA GTC AAC AAG GAC GAG GGC
Ser Gly Asn Phe Lys Lys Thr Gln Ile Leu Leu Gly Val Asn Lys Asp Glu Gly
311
            1036                1051                1066
TCG TTT TTC CTC TTG TAC GGA GCG CCG GGT TTC AGC AAG GAC TCT GAA AGC AAA
Ser Phe Phe Leu Leu Tyr Gly Ala Pro Gly Phe Ser Lys Asp Ser Glu Ser Lys
329
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1081                1096                1111                1126
ATC TCT CGG GAA GAC TTC ATG TCA GCG GTC AAG CTA AGC GTT CCC CAC GCC AAT
Ile Ser Arg Glu Asp Phe Met Ser Ala Val Lys Leu Ser Val Pro His Ala Asn
347
       1141                1156                1171                1186
GAC TTA GGG TTG GAC GCT GTC ACG CTA CAA TAC AGA GAC TGG ATG GAT GAC AAT
Asp Leu Gly Leu Asp Ala Val Thr Leu Gln Tyr Thr Asp Trp Met Asp Asp Asn
365
               1201                1216                1231
AAT GGT ATA AAG AAC AGA GAT GGA TTG GAC GAC ATC GTA GGG GAC CAC AAC GTC
Asn Gly Ile Lys Asn Arg Asp Gly Leu Asp Asp Ile Val Gly Asp His Asn Val
383
    1246                1261                1276                1291
ATA TGC CCC TTG ATG CAC TTT GTT AAC AAG TAC ACC AAG TTT GGC AAT GGC ACC
Ile Cys Pro Leu Met His Phe Val Asn Lys Tyr Thr Lys Phe Gly Asn Gly Thr
401
               1306                1321                1336
TAC CTG TAC TTC TTC AAC CAC CGA GCC TCA AAC CTG GTG TGG CCG GAG TGG ATG
Tyr Leu Tyr Phe Phe Asn His Arg Ala Ser Asn Leu Val Trp Pro Glu Trp Met
419
1351                1366                1381                1396
GGC GTC ATC CAC GGC TAT GAG ATT GAG TTC GTC TTC GGG CTG CCT CTG GTG AAG
Gly Val Ile His Gly Tyr Glu Ile Glu Phe Val Phe Gly Leu Pro Leu Val Lys
437
       1411                1426                1441                1456
GAG CTG AAC TAC ACA GCG GAG GAG GAA GCG CTG AGC CGG AGG ATA ATG CAT TAC
Glu Leu Asn Tyr Thr Ala Glu Glu Glu Ala Leu Ser Arg Arg Ile Met His Tyr
455
               1471                1486                1501
TGG GCG ACA TTC GCA AAG ACT GGA AAC CCA AAC GAA CCC CAC TCA CAG GAG AGC
Trp Ala Thr Phe Ala Lys Thr Gly Asn Pro Asn Glu Pro His Ser Gln Glu Ser
473
    1516                1531                1546                1561
AAA TGG CCT CTC TTC ACT ACC AAG GAG CAG AAA TTT ATT GAC CTC AAC ACA GAA
Lys Trp Pro Leu Phe Thr Thr Lys Glu Gln Lys Phe Ile Asp Leu Asn Thr Glu
491
            1576                1591                1606
CCC ATG AAA GTC CAC CAG CGA CTC CGA GTT CAG ATG TGC GTG TTC TGG AAC CAG
Pro Met Lys Val His Gln Arg Leu Arg Val Gln Met Cys Val Phe Trp Asn Gln
509
1621                1636                1651                1666
TTC CTC CCC AAG CTC CTC AAC GCC ACA GAG ACC ATT GAT GAG GCA GAA CGC CAG
Phe Leu Pro Lys Leu Leu Asn Ala Thr Glu Thr Ile Asp Glu Ala Glu Arg Gln
527
       1681                1696                1711                1726
TGG AAG ACG GAG TTT CAT CGG TGG AGT TCC TAC ATG ATG CAC TGG AAG AAC CAA
Trp Lys Thr Glu Phe His Arg Trp Ser Ser Tyr Met Met His Trp Lys Asn Gln
545
               1741                1756                1770        1780
TTT GAC CAC TAC AGC AGA CAC GAG AGC TGT GCT GAG CTG TGA GCTCTGCTCT GCAGGTCGCC
Phe Asp His Tyr Ser Arg His Glu Ser Cys Ala Glu Leu
563
        1790        1800        1810        1820        1830        1840        1850
TGGTGAAGCA GAGAGCAGAG TCCGATATTG ACCAGACACC CAGTCTAGTT CCTGGAGACC CTGCCTGCC

        1860        1870        1880        1890        1900        1910        1920
CTCTGAGCCC CCCCCCCCCA CCCCGCCAAA CCTCGCCTTC GAGACTGCCT CCCTCCCATC CCTCCTCTAA

        1930        1940        1950        1960        1970        1980        1990
CCCCAGGCTG CCGACCTCGT CTCTCGCACC GTCACTCGTA AATCTGCGGC ACCAATCTTT CGGCCACCCA

        2000        2010        2020        2030        2040        2050        2060
CTCTCCCATC AGCCCAACTC TCTCCTTCCT CTTCCCCACC ACTACCCCCC TATTACCCAC TCTCCACCTG

        2070        2080        2090        2100        2110        2120        2130
CCGCACTCTC TCTCCCAATA CCGTCAGCAA CCATTCCAAT CCCCTAAATC TCTCTCCTGT CACCTATTGA

        2140        2150        2160        2170        2180        2190        2200
CGTTCTACCC CCCCCCCCTC ACTCTGTCTC CCCAAGTACT CCCTCCCCGT TCATCCCATA GTTCCCAACT

        2210        2220        2230        2240        2250        2260        2270
CCCCAAAAGA CTTCTGGTCT CCTTCCTAAG TGGCCCTTCA CCTGTACAAA TCTGGGTGAG AGCCAATATG

        2280        2290        2300        2310        2320        2330
ATCCTGGCTG ATTCTCTGCT TTATCCAGTT TCCTTGCCCA ATCATTAGAT CCTCCAACTC GAATTC
```

Figure 3. Complete amino acid sequence of Human Serum Cholinesterase (35).

catalytic site. Wilson and his co-workers in 1951 proposed a schematic model of the active site which is composed of two subsites: an anionic and an esteratic site (45). The most important aspect of the anionic site is that it contains one or, more likely, several negatively charged groups which interact with the positively charged quaternary nitrogen of acetylcholine. These Coulombic forces are supplemented by hydrophobic interactions of the methyl groups with a tryptophan residue which has been shown to be present by the observation that a charge-transfer complex is formed between the enzyme and the aromatic cation N-methylacridinium (45,28). In general the anionic site determines the specificity of the enzyme with respect to the alcohol moiety, the combined interactions serving to orient the substrate such that its carbonyl group is adjacent to the esteratic site, which is the one actually involved in the catalytic process.

A key element of the esteratic site is a serine residue of enhanced nucleophilicity. The importance of this residue, now known to be Ser^{200} (46-49), was first evident from studies on the inactivation of AChE by organophosphates which showed that inactivation was accompanied by the phosphorylation of a single Ser residue. The use of ^{3}H-diisopropylfluorophosphate (DFP) allowed the isolation of labelled peptides from the active site region and showed unambiguously that the labeled moiety was the serine hydroxyl group.

Studies of the pH-dependence of the rates of AChE-catalyzed reactions by Krupka (46,50) revealed a bell-shaped dependence from which apparent pK_a values of 6.5 and 10.5 could be extrapolated. The lower ionization corresponds to the pK_a of the imidazolium group of histidine and suggests a catalytic role for the unprotonated form of

this group. The results of these and many other kinetic studies too numerous to document here, have culminated in the generally-accepted view that the AChE-catalyzed hydrolysis of acetylcholine proceeds through an acylenzyme mechanism involving nucleophilic (serine) and general acid-base (histidine) components, as shown in Figure 4.

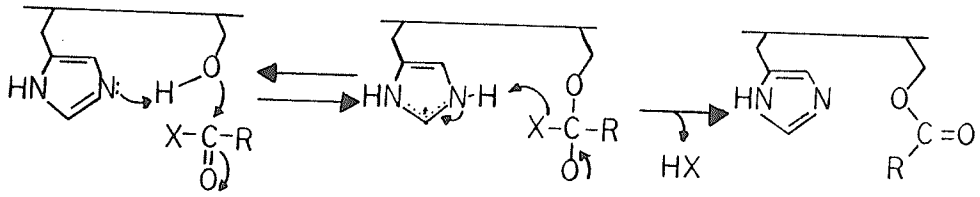
Many of the molecular details of the mechanism of this reaction are derived by analogy with the closely similar pathway of ester hydrolysis by other serine esterases such as chymotrypsin. X-ray crystallographic studies on the latter (51) led to the identification of a set of residues referred to as the "catalytic triad" and consisting of spatially related Ser, His and Asp residues positioned in such a way as to enhance the nucleophilicity of the serine hydroxyl group *via* a so-called charge-relay mechanism. As yet, there is no evidence for the existence of such a system in the cholinesterases.

A comparison of the active site sequences of AChE from *T. californica* and BChE shows considerable homology in the active site region. This would suggest that both BChE and *T. californica* AChE react in the same way with respect to the active site. This however, is not the case. It has been previously demonstrated that the rate of hydrolysis of substrate for *T. californica* AChE decreases with respect to the length of the acyl chain, and that the rate of hydrolysis of substrate for BChE increases with the length of the acyl chain. Other differences such as the effects of inhibitors, ions and excess substrate have been attributed to differences in and around the active site.

It has generally been accepted that *T. californica* AChE contains both an esteratic and anionic site, but up until 1966, the question of whether or not BChE actually contained an anionic site had not been

Figure 4. Proposed mechanism of action of serine esterases depicting the tetrahedral and acyl enzyme intermediates (44).

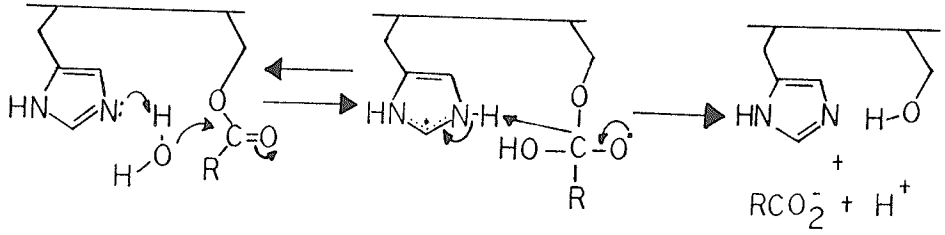
ACYLATION



TETRAHEDRAL INTERMEDIATE

ACYLENZYME

DEACYLATION



TETRAHEDRAL INTERMEDIATE

resolved. The differences in behaviour resulted in a variety of different opinions regarding the presence or absence of an anionic site in BChE. Some workers favoured the idea that BChE possessed only an esteratic site, while others believed that BChE like AChE possessed both an anionic site and an esteratic site. Still others suggested that BChE contained one esteratic site and one anionic site whereas *T. californica* AChE contained one esteratic site and two anionic sites (52).

A comparative study of the interactions of *T. marmorata* AChE and human serum cholinesterase with carbinol acetates of pyridine and N-methyl pyridine was carried out by Augustinsson in 1966 in order to determine the presence or absence of an anionic site (53). He observed that the introduction of a charge on the nitrogen of pyridine resulted in an increase in the rate of hydrolysis of butyrylcholine by BChE and a decrease in the rate of hydrolysis of butyrylcholine by AChE. With acetylcholine as a substrate, the quaternary carbinols inhibited BChE but not AChE. From these results, he concluded that there was indeed a "non-esteratic" site present in BChE in addition to the esteratic site. BChE also exhibited the same pH-activity relationships for both the tertiary and quaternary compounds, whereas AChE showed a different profile for each class of compounds. From this it was concluded that coulombic attractions were predominant in AChE and that in BChE, Van der Waals forces are predominant.

Kabachnik *et al.*, in 1970, attempted to account for the differences in the behaviour of AChE and BChE through the use of a series of organophosphorus inhibitors (54). The behaviour of the enzymes towards these agents have led Kabachnik to conclude that there are hydrophobic regions

which surround the anionic site. It is these regions which appear to be responsible for the differences in the behaviour of the enzymes towards the substrates.

Acetylcholinesterase has been shown to catalyze the hydrolysis of a wide variety of compounds. Among these are aryl esters, anilides, thioesters, amides, selenoesters, acyl homologues, and N-demethylated analogues of acetylcholine (44). The best substrates reported for AChE are acetylcholine, acetylthiocholine and acetylselenocholine. The relative second order acylation rate constants, k_E, for these compounds are 1.0, 1.58 and 3.62, (28,55) respectively. Given that k_E for the natural substrate acetylcholine is about 1.6×10^8 $M^{-1}s^{-1}$, the rate determining step in these reactions evidently is the diffusion of substrate to the catalytic site. In addition, the turnover number of in excess of 10^4 s^{-1} is a further illustration of the remarkable catalytic efficiency of AChE (44).

The wide variety of compounds which can be hydrolyzed by AChE provides a large selection of alternative substrates which have proven useful in kinetic studies. Particularly noteworthy is acetylthiocholine (ASCh), whose introduction by Ellman *et al.* (56) provided a very convenient spectrophotometric means of carrying out steady state kinetic studies. The pre-steady state behaviour of AChE can be conveniently examined through the use of the fluorogenic carbamoylating agent N-methyl-(7-dimethylcarbamoyloxy)quinolinium iodide (M7C) (28,57, 58). This compound carbamoylates the active Ser of acetylcholinesterase in a relatively slow acylation phase during which a stoichiometric "burst" or release of the fluorescent hydrolysis product N-methyl-7-hydroxyquinolinium occurs, thereby providing an estimate of the

catalytic site concentration (28). Both of these pseudosubstrates have been extensively used in the present study; the pertinent reactions are shown in Figure 5.

v) Peripheral anionic sites

The ability of AChE to undergo ligand-induced conformational changes was first suggested by Changeux (59) who in 1966 observed that inhibition of *Torpedo marmorata* AChE by gallamine was not strictly competitive in nature. The finding that gallamine could relieve inhibition by decamethonium and other bisquaternary inhibitors led to the proposal that AChE possesses "peripheral" anionic sites, distinct from the catalytic site, whereby binding of cationic ligands could influence the catalytic properties of the enzyme. Changeux also noted that the effects of many pharmacologically significant compounds could be correlated with their activities at the ACh receptor. Non-depolarizing receptor blockers (pachycurares) were generally found to be noncompetiive inhibitors of AChE and antagonistic towards inhibition by depolarizing drugs (leptocurares). Furthermore, inorganic cations (Na^+, Ca^{2+}, and Mg^{2+}) were found to increase V_{max} and Km for ACh hydrolysis, whereas the nondepolarizing inhibitors decrease both V_{max} and Km (129). Based on these results, Changeux suggested two conformational states for AChE, P and D, where P is the state stabilized by the non-depolarizing inhibitors and D is the state which is stabilized by the depolarizing receptor blockers and high salt concentrations.

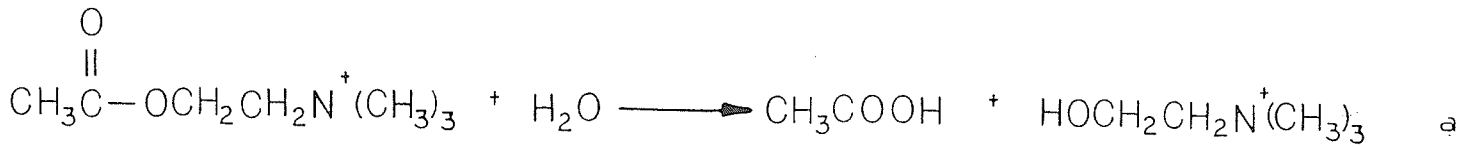
Belleau *et al.* used mono and bisquaternary ligands to study the

Figure 5. Substrate and pseudosubstrate hydrolysis by AChE.

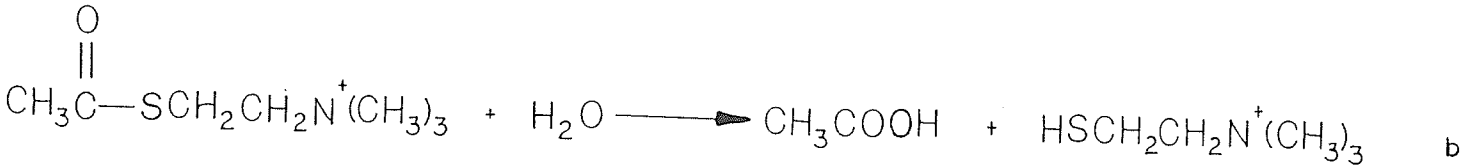
a: acetylcholine, the natural substrate.

b: acetylthiocholine, a useful pseudosubstrate in steady-state kinetic studies.

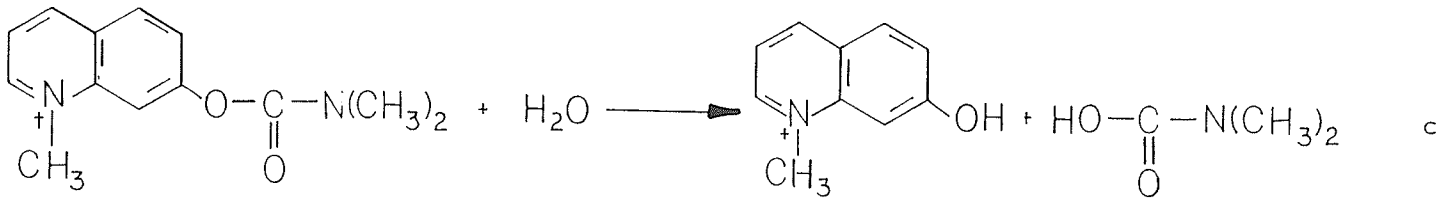
c: N-Methyl-(7-dimethylcarbamoylxy)quinolinium Iodide (M7C), a fluorogenic carbamoylating agent useful in pre-steady-state kinetic studies.



a



b



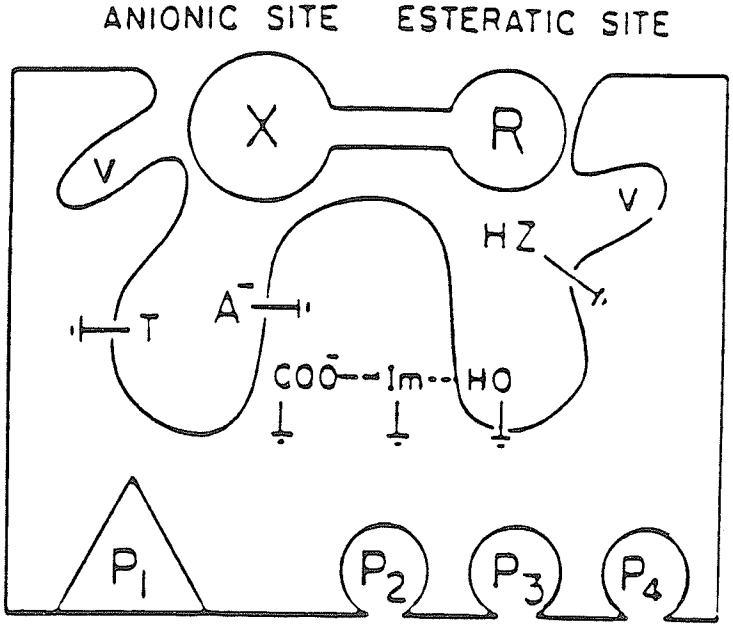
c

acceleration of methanesulfonylation of AChE by methanesulfonyl fluoride (60). The enzyme bound 4 mols of either of the two types of ligand per tetrameric enzyme molecule. Acceleration was maximal with decamethonium, a leptocurare, suggesting that this compound binds to the enzyme by spanning both the anionic subsite of the catalytic site and a second anionic site approximately 1.0-1.4 nm away. In contrast, pachycurare drugs were inhibitors of methanesulfonylation, thus providing further support for the two-state model of Changeux as well as for the existence of peripheral anionic sites on the enzyme surface.

The existence of a multiplicity of peripheral sites was suggested by the work of Roufogalis and Quist (61). They demonstrated that the antagonism between Ca^{2+} (which is an activator of AChE) and decamethonium was essentially competitive whereas that between gallamine and decamethonium was complex and primarily noncompetitive. On the other hand, Ca^{2+} and gallamine did not compete at all. Subsequently, Roufogalis and Wickson (62-63) showed that treatment of AChE with water-soluble carbodiimides, which modify carboxyl groups, abolished the stimulating effects of both Ca^{2+} and gallamine and caused a ten-fold increase in the inhibition constant of decamethonium. On the basis of these and other results, a model for the distribution of anionic sites on the AChE subunit was proposed by Rosenberry (28) and is shown in Figure 6. In this model, the peripheral site is regarded as a group of anionic subsites having different ligand selectivities. Site P_1 binds Ca^{2+} and one of the quaternary ammonium groups of decamethonium (with the other binding to the anionic subsite of the catalytic site 1.0-1.4 nm away). The designation of the other group of sites as P_2, P_3, and P_4 is based on the presence of three quaternary

Figure 6. The distribution of sites on the acetylcholinesterase subunit (28).

The substrate is represented by RX. X is the leaving group and R is the acyl group. V represents hydrophobic regions believed to be present in the vicinity of the catalytic site. One of these residues is tryptophan (T). A^- defines one or more negatively charged groups at the anionic site. HZ represents an acidic group present at the esteratic site. P_1-P_4 represent peripheral anionic sites, and COO^--HIm-HO represents the postulated charge relay system involved in catalysis.


ANIONIC SITE
ESTERATIC SITE
X
R
V
V
HZ
T
A⁻
COO⁻
Im
HO
P1
P2
P3
P4

ammonium groups in the gallamine structure. Larger ligands such as d-tubocurarine may span the entire P_2-P_4 region.

The kinetic consequences of peripheral site occupancy were explored in a series of studies by Tomlinson *et al.* (64-67). Propidium, a fluorescent probe of peripheral sites (see below), first introduced by Taylor *et al.* (68), was found to competitively antagonize the binding of gallamine. Both ligands were found to increase V_{max} and K_m for ASCh hydrolysis under low ionic strength conditions, resulting in the generation of curved Lineweaver-Burk plots. Increasing the ionic strength to 0.1 with NaCl also results in the activation of the enzyme. A similar, but more specific, effect was found with Ca^{2+} and Mg^{2+} at 0.1-1 mM concentrations. Pre-steady state kinetic studies using the active site carbamoylating agent M7C showed that the effects of the Group IIA cations was confined to the deacylation phase of the reaction whereas the organic cations propidium and gallamine increased both acylation and deacylation rates. These studies provided additional support for the delineation of P_1 and P_2-P_4 subsites and the existence of ligand-dependent "active" and "activated" states of AChE.

A completely different effect was observed when AChE from *T. californica* (69) or *E. electricus* (65,66) was exposed to Group IIB cations (Zn^{2+}, Cd^{2+}, and Hg^{2+}). These cations, as well as the organic cation d- tubocurarine, were found to promote a slow conversion of the enzyme to a third, "unreactive" state as evidenced by the progressive loss of the M7C carbamoylation amplitude but with little effect on the actual carbamoylation rates. The metal ion-induced inactivation could be reversed on addition of chelating agents such as EDTA; however, the amount of recoverable activity decreased as the period of preincubation

with metal ions was increased, suggesting that the "unreactive" state might be an intermediate in a pathway leading to irreversible denaturation of the enzyme. The observation that exclusion of oxygen from the medium delayed the irreversible denaturation was an important factor in determining the objectives of the present study (see below).

Further support for the three-state model for AChE came from studies with La^{3+} (67). This cation resembles Ca^{2+} in its binding selectivity, yet is also a heavy metal ion which might be an inactivator of AChE. Indeed, it was found that La^{3+} promotes both activation (fast) and inactivation (slow) under low ionic strength conditions. The titration curve for inactivation was biphasic and extended over several orders of magnitude of cation concentration, suggesting that inactivation can arise from both active and activated conformations of the enzyme. At high ionic strength, when the enzyme exists entirely in the activated state, the titration curve was monophasic and showed normal concentration dependence.

In summary, the accumulated kinetic evidence discussed above firmly establishes the existence of peripheral sites on AChE to which the binding of a variety of cationic ligands can modulate the properties of the catalytic site. Further characterization of the various binding sites on the enzyme surface has been obtained through the use of site-specific fluorescent probes, to be discussed in the next section.

vi) Probes of acetylcholinesterase function

Kinetic studies have been the major experimental tool for the determination of potential peripheral sites in AChE, the inhibition

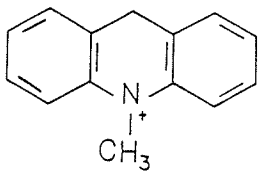
patterns obtained with quaternary ammonium ligands and inorganic cations being strongly suggestive that these compounds interact strongly with AChE at more than a single site (28,65). The use of NMR spectroscopy has recently supported the existence of a specific binding site for atropine, distinct from the active site (70,71). While both these methods are useful, the first is dependent upon the influence of multiple catalytic parameters, while NMR requires a high concentration of protein, which is not always available.

The properties of fluorescent compounds which bind to specific sites on the protein make them good indicators of the various types of interactions in which AChE can participate. The major advantage of fluorescent ligands is that they increase the sensitivity of the measurements, which allows a decrease in the amount of protein used. These ligands can also measure the influence of various inhibitors and modifying agents on protein conformation, as the fluorescence properties of the probes are sensitive to their surrounding microenvironment (28).

To date, two types of fluorescent ligand-AChE complexes have been used in fluorescence studies. One type involves ligands which have high quantum yields in solution, and low quantum yields upon association with the enzyme. The second type of ligands used have a low quantum yield in solution which is greatly enhanced upon binding to the enzyme (72,73). The structures of some compounds that have been used in the study of AChE-ligand interactions are shown in Figure 7.

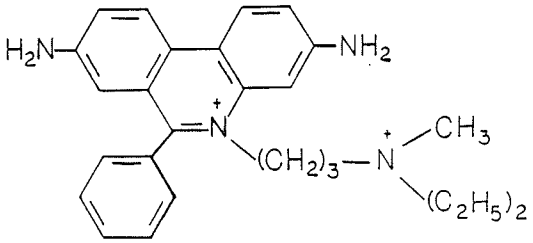
N-methylacridinium iodide (N-MAC) and bis(3-aminopyridinium)-1,10-decane diiodide bind reversibly to the active sites. Binding was demonstrated by the decrease in the quantum yield of the bound compound

Figure 7. Some fluorescent probes used in the study of AChE.



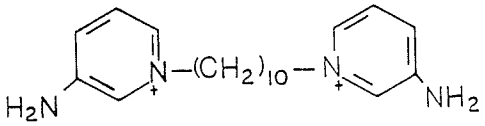
a

N-METHYLACRIDINIUM



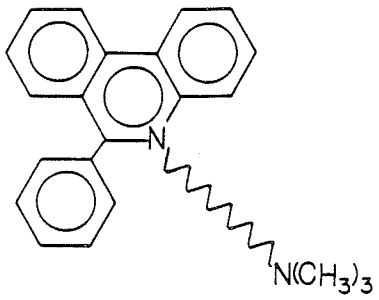
b

PROPIDIUM



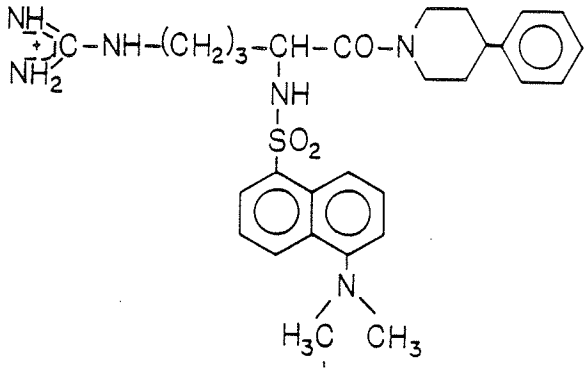
c

BIS(3-AMINOPYRIDINIUM)-1,10-DECANE



d

DECIDINIUM



e

N^{α}-DANSYL-L-ARGININE-4-PHENYLPIPERIDINE-AMIDE
(DAPPA)

relative to that of the compound free in solution. This quenching of fluorescence can be attributed to the sensitivity of the probe to the surrounding environment on the protein surface. Through the method of continuous variations, it was shown that ligand binding reaches a maximum stoichiometry at a ratio of 1:1 AChE to ligand. Both of the fluorescent probes overlap the catalytic sites, as was determined from the similarities in their competitive inhibition constants (ACh as substrate) and their dissociation constants determined by direct titration. The inhibition of acetylcholine hydrolysis by both compounds shows both competitive and noncompetitive components in Lineweaver-Burk plots. The latter, in conjunction with the complete quenching of the fluorescence of the bifunctional bis(3-aminopyridinium)-1,10-decane compound, provides additional evidence for the existence of a second anionic binding site on the enzyme surface, approximately 14 Å from the catalytic site. The observed fluorescence quenching can possibly be attributed to the presence of hydrophobic groups on the protein, surrounding the anionic binding sites, as both compounds exhibit weak fluorescence in solvents with low dielectric constants. The ability of the probes to be displaced by nonfluorescent ligands of AChE, makes them very useful in the study of the binding interactions of a variety of other compounds bound at the active and peripheral sites (74-76).

Propidium (3,8-diamino-5,3'-diethylmethylamino-n-propyl-6-phenyl-phenanthridium), like all the other fluorescent probes used to date, is a potent inhibitor of AChE. Propidium exhibits a 10-fold increase of fluorescence intensity and a 1:1 stoichiometry when bound to *T. californica* AChE (77). This probe is unique in that it binds exclusively to a peripheral anionic site, as indicated by the fact that

its binding to the enzyme is unimpaired by the presence of edrophonium, an active site-directed competitive inhibitor, or by methanesulfonylation of the active site serine residue. Ligands with some peripheral-site selectivity such as gallamine and d-tubocurarine, as well as bisquaternary ligands such as decamethonium, which interacts with both sites, are effective inhibitors of propidium binding (77,78).

Decidinium is also a fluorescent bisquaternary ligand which binds to the native enzyme (79). This ligand was introduced in order to determine whether the active site of AChE indeed contains an anionic subsite or simply is a hydrophobic trimethyl binding site. *T. californica* AChE was inhibited by isosteric β-(trimethylamino)ethyl- and (3,3-dimethylbutylmethyl)-phosphonofluoridates, which mimic the tetrahedral intermediate in the acylation stage of catalysis on reaction with the enzyme. If the anionic site is uncharged, then the quaternary ammonio and trimethyl functions of the phosphonylenzymes should occupy the same site. However, parallel experiments using a variety of ligands bound to the phosphonylated and native AChE show that this is not the case. The fluorescent probe decidinium binds to the native enzyme such that the aromatic moiety binds to the peripheral site and the trimethylamino function binds at the active site. The dissociation constant of the dimethylbutylmethylphosphono-AChE complex with decidinium (Kd=54 nM) is nearly the same as that for the complex with the native enzyme (Kd=21 nM) , whereas the charged methylphosphono-AChE complex has a 100-fold lower affinity for decidinium (Kd=4.3μM). These results are consistent with the occupation of the anionic subsite of the active site in the charged methylphosphono-AChE complex, but not in the uncharged complex. This is supported by the fact that some n-

alkyl mono- and bisquaternary ligands bind with the same affinity to the native enzyme and to dimethylbutylmethylphosphono-AChE, whereas pyrenebutyl methylphosophono- AChE binds these ligands with 100 fold lower affinity relative to the binding to native AChE. The large pyrenebutyl group presumably intrudes into the anionic subsite (80).

N-dansyl-L-arginine-4-phenylpiperidine amide (DAPPA)is a relatively new fluorescent probe which has been used to assist in further defining the topography of horse serum cholinesterase. DAPPA, like a variety of other probes is a potent competitive inhibitor of this enzyme. Fluorescence titrations and inhibition studies over a concentration range of 0.01 to 0.03 micromolar have determined the dissociation constant to be 0.011-0.013 micromolar and 0.016 micromolar respectively, which indicated the probe to be tightly bound to the protein. DAPPA exhibits a 42-fold increase in the levels of fluorescence when bound to the protein in a 1:1 mole ratio as compared to the fluorescence level of free DAPPA. It has a calculated extinction coefficient of 4.4 mM, which makes it very useful for studies in the micromolar concentration range. DAPPA is also unique in that it is an arginine analog, which has proven to be particularly useful in the study of structure-activity relationships among arginine derivatives and thrombin, a serine protease.

Based on previous data regarding the use of arginine derivatives in the determination of the active surface of thrombin, dansylarginine derivatives were prepared by the substitution of the carboxyl side of arginine, and the inhibitory effects on the activity of BChE were measured. The possible similarity between thrombin and BChE i.e., the existence of a hydrophobic pocket located in the vicinity of the esteratic subsite and a peripheral site next to the hydrophobic site,

was examined in an attempt to determine the presence or absence of such sites in BChE. The substitution effects showed an increase in inhibitory activity as the chain length increased from ethylamide to butylamide, and from piperidine to ethylpiperidine. The inhibitory activity was further increased upon substitution of the 4-ethyl group of piperidine for 4-phenylpiperidine. These results indicated that BChE has a hydrophobic pocket corresponding to ethylpiperidine. The strong binding and competitive action exhibited by DAPPA is explained by assuming the arginine side chain interacts with the anionic subsite and that the CO group is in the proximity of the active serine, and the piperidine ring being accomodated by the hydrophobic pocket near the esteratic site.

This hydrophobic pocket has an overall negative charge, as the introduction of a negative charge in the 2-position of piperidine caused a decrease in the inhibitory activity towards BChE. Furthermore esterification of the charged carboxyl group restored the inhibitory activity to the same level of the compound without the carboxyl group. This is indicative of there being an entity on the opposite side of a the hydrophobic binding pocket which repulses the negative charge of the carboxyl group.

The use of these fluorescent ligands has shown that there are indeed peripheral sites present in AChE. Fluorescent studies are potentially very useful in the sense that the interactions can be measured directly and with high sensitivity. Their use as probes of ligand-induced conformational changes of AChE is, however, limited by the fact that the probes bind noncovalently to the enzyme and in many cases are displaced by other ligands, leading to loss of signal. The

finding in our laboratory that some species of cholinesterase contain a free sulfhydryl group opens up the possibility of developing fluorescent probes that are covalently attached to the protein where they might serve as more useful reporter groups in the presence of added ligands.

vii) Sulfhydryl groups in AChE and BChE

Acetylcholinesterase was originally considered to be a sulfhydryl enzyme, on the basis that it could be inhibited by a variety of classical thiol-specific inhibitors (82). However, the concentration of thiol-specific reagent required for the inhibition of AChE was very high, and the rates of inactivation very low, when compared to other thiol containing enzymes. Various studies conducted to determine the sulfhydryl nature of the cholinesterases showed no evidence for the participation of SH groups in AChE-catalyzed reactions (28,83-85). However, there was also no strong evidence against the presence of a possible nonessential thiol group (86). Evidence which suggested that there might be a nonessential thiol group present in AChE was first obtained in our laboratory (66,82).

Experiments performed using *T. californica* AChE in the presence of inactivating metals such as Zn^{2+}, Cd^{2+}, and Hg^{2+} showed that the inactivation could be reversed upon addition of chelating agents such as EDTA. However, the amount of recoverable activity declined in proportion to the time of exposure of the enzyme to the metal ion prior to addition of EDTA. The fact that this irreversible inactivation occurs more rapidly under oxidizing conditions suggested the possible

involvement of a nonessential thiol group in the process (86). Initial attempts to assay these thiol groups with the reagent DTNB under denaturing conditions were not very successful. There was a detectable reaction, but the protein concentration was too low for an accurate assessment of the thiol content.

The possible presence of a free thiol group was also suggested when it was observed that AChE was able to react with the thiol-selective alkylating agent N-dansylaziridine. Although a component having thin layer chromatographic mobility to that of S-dansylaminoethylcysteine could be demonstrated in hydrolysates of the labeled protein, there were also several other fluorescent spots present indicative of alkylation of other groups on the protein (66). This, and the fact that an excessive reaction time was required (17 hours), led to a search for a more specific thiol reagent.

SH groups are by far the most reactive groups in proteins, and are usually the ones which are the most easily modified. There are a great many reagents which will react with proteins, and they have been the subject of many reviews. The ones which are specifically classified as SH specific fall into three categories: 1) mild oxidizing agents, such as ferricyanide, pophyrindin, iodine, iodosobenzoate; 2) alkylating agents such as iodoacetamide, iodoacetate, and N-ethylmaleimide; and 3) mercaptide forming organometallic compounds such as p-mercuribenzoate and trivalent organic arsenicals (87,88). The major drawbacks to most of the oxidizing reagents are that, unless conditions are ideal, groups other than the SH will be attacked, and furthermore, since the oxidation often involves the formation of a disulfide pair, the reaction is dependent upon the proximity of the SH bonds in the protein

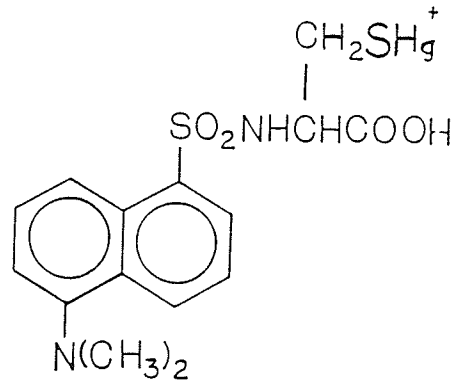
(88). The use of alkylating agents such as iodoacetic acid and iodoacetamide is often not very informative, as these reagents lack specificity, and the reactions are not easily reversible. The use of mercurials as labeling agents has increased the specificity for SH groups. As well as a high affinity for SH groups, they react under mild conditions and the reaction can be reversed upon the addition of an excess of a simple mercaptan. Organomercurials also react with a single SH group, and with SH groups which appear to be hidden to other reagents (72,87,88). In all cases, quantitation of the number of modified groups can be difficult.

The availability of fluorescent reagents such as didansylcysteine (89,90), iodoacetamidenapthylaminesulfonate (I-AEDANS) (91), dansylaziridine (81,92), difluorescein cysteine (91,95), fluorescein mercuric acetate (90,94), mercurichrome (97), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (98), N-(1-anilinonapthyl-4)-maleimide (ANM) (99), N-(p-(2-benzimidazolyl)phenyl)-maleimide (BIPM) (100) and pyrene maleimide (101), overcomes the sensitivity and quantitation problem to a large extent. These reagents, while they have been highly useful for certain protein systems, also have their disadvantages. For example, the compounds dansylaziridine, ANM, and pyrenemaleimide all have limited solubility in aqueous solutions. Many lack specificity, require a long reaction time, and excess reagent may be required for successful reaction. ANM, I-AEDANS, BIPM, pyrenemaleimide, and dansylaziridine have complex syntheses, and in some cases have limited stability. Other problems such as low quantum yields, heterogeneous fluorescence spectra, and changes in the spectra at neutral pH, make these probes difficult to use.

The compound S-mercuric-N-dansyl-cysteine (SMNDC), was first introduced by Leavis and Lehrer in 1973 (102). The compound appears to possess many advantages over most other fluorescent sulfhydryl reagents. The dansyl moiety allows efficient energy transfer from the protein to the probe, and the fluorescence is sensitive to the protein environment and has a long lifetime. The organomercurial nature of the compound provides for a high affinity for SH groups, it is easily prepared and is stable and water soluble. It has the potential ability to react rapidly, and stoichiometrically with SH groups over a wide range of pH values, and the reaction is readily reversed by addition of excess low molecular weight thiol compound such as DTT. The structure and properties of SMNDC are shown in Figure 8.

Various experiments conducted with this probe have shown that Troponin C, and Tropomyosin, Actin, (102) and Ca^{2+}-ATPase (103) Na^{+}/K^{+}-ATPase (104), Galactosyltransferase (105), and Thiolase I (106) react with SMNDC through formation of mercaptide linkages. In many cases, the high affinity of the probe for the enzyme permitted the direct titration of the number of free SH groups present in the protein. This reagent thus appeared to be of great potential use in the search for a free SH group in AChE and BChE. Preliminary experiments conducted by Mutus *et al.* (82) established that it indeed binds tightly to AChE, as evidenced by the retention of the label by the protein during SDS-PAGE under nonreducing conditions. The evaluation of SMNDC as a potential probe of SH groups in AChE and BChE forms the basis of the present study.

Figure 8. Structure of S-Mercuric-N-Dansyl-Cysteine (SMNDC) and its reaction with protein bound SH groups.



S-MERCURIC-N-DANSYLCYSTEINE

$$R\text{-}SH \;+\; Hg^{+}SCH\text{-}R'\;Cl^{-} \longrightarrow RSHgSCHR' \;+\; HCl$$

viii) Objectives of this study

Given the evidence for the possibility of a free sulfhydryl group, as provided by previous work done in our lab, the work in this thesis revolves around the evalutation and subsequent use of the SMNDC as a potential probe of free SH groups in *T. californica* AChE, and human serum cholinesterase.

The first step was to establish conditions and criteria for the delineation of noncovalent and covalent interactions of the probe with proteins. To this end, a number of test proteins containing 0, 1, and 2 SH groups were used in the reaction with SMNDC.

The second step was the application of these criteria to AChE, to establish whether or not a covalent or tight noncovalent bond existed.

The third aspect of this project involved the purification of BChE for the purpose of determining the presence or absence of a free SH group in this protein.

The fourth aspect was the study of labeled AChE and labeled BChE to determine whether or not the modified enzyme retained catalytic activity and to determine if SMNDC might have potential use for the monitoring of ligand induced conformational changes.

MATERIALS

Materials	Supplier
crude 11s *E. electricus* AChE (Type VI-S)	Sigma Chemical Company St. Louis, Missouri, U.S.A.
Acetylthiocholine chloride	
Acetylcholine chloride	
Benzoylcholine chloride	
Cbz-ε-aminocaproic acid	
Decamethonium bromide	
Didansylcysteine	
DTNB	
EDAC	
Electrophoresis standards (MW-SDS-200)	
gallamine triethiodide	
Isobutylchloroformate	
N,N-dimethyl-m-phenylenediamine	
N,N-dimethyl-p-phenylenediamine	
Procainamide hydrochloride	
Tris(hydroxymethyl)aminomethane	
Trypsin (Type III)	
Trypsin inhibitor (soybean)	
d-Tubocurarine	

Materials	Supplier
Acrylamide Ammonium persulfate Dithiothreitol N,N,N′,N′-tetramethylethylenediamine Methylene-bis-acrylamide Protein assay kit Sodium dodecyl sulfate	Bio-Rad Laboratories (Canada) Ltd. Mississauga, Ontario
Dimethylcarbamoyl chloride Methyl iodide	Aldrich Chemical Company Milwaukee, Wisconsin, U.S.A.
Hydrogen bromide (anhydrous)	Matheson Gas Products Whitby, Ontario, Canada
Edrophonium chloride (Lot # 044033)	Hoffman - La Roche Inc. Nutley, New Jersey, U.S.A.
Torpedo californica electroplax tissue	Pacific Biomarine, Venice, California, U.S.A.

Materials	Supplier
Amicon PM10 membranes CX-10 ultrafiltration apparatus Pressure dialysis equipment	Amicon Canada Ltd. Oakville, Ontario,
7-hydroxyquinoline N-methyl-7-(dimethylcarbamoyloxy)-quinolinium iodide (M7C)	Eastman Kodak Co. Rochester, New York, U.S.A.
Ethanol Sucrose Glycine Hydrochloric acid Sodium hydroxide Sodium chloride Phosphorus pentoxide Sodium azide Monobasic and dibasic sodium phosphate Volumetric pipette tips Polystyrene cuvettes	Fisher Scientific Winnipeg, Manitoba, Canada

Buffers and Reagents

1) Ellman assay buffer : 0.10 M NaH_2PO_4, pH 8.0.

2) Equilibration buffer (EQB) : 0.010 M NaH_2PO_4/ 0.10 M NaCl, pH 7.4.

3) Torpedo preparation buffer (TPB) : 0.01M $NaHCO_3$/0.1M NaCl/0.04M $MgSO_4$, pH 7.4.

4) Serum dialysis buffer : 0.02M Na acetate/1.0mM EDTA, pH 4.0

5) Low ionic strength Tris buffer : 1.0mM Tris, pH 7.5

6) High ionic strength Tris buffer : 10.0mM Tris/0.1M NaCl, pH 7.5

7) Electrophoresis buffer : 0.04M Tris acetate/0.2% SDS, pH 7.4.

8) Benzoylcholine assay buffer : 0.067M Na^+/K^+ phosphate, pH 7.4.

9) 5,5'-dithiobis-(2-nitrobenzoic acid) : 0.010 M DTNB in Buffer 1)

10) Acetylthiocholine (ASCh) : 0.0764 M in H_2O, prepared fresh every 4 days.

All buffers used in these studies were prepared from glass-distilled deionized water and then ultrafiltered through Millipore Type HA (0.45 μm pores) filters.

SDS-PAGE Gels

10% SDS-polyacrylamide tube gels were prepared as follows:

	amount used (mL)
30% acrylamide, 0.8% bis acrylamide solution	8.33
1.5M Tris-Cl pH 8.8	6.25
20% SDS	0.13
water	9.64
ammonium persulfate 1.5%	0.60
TEMED 100%	0.025

The TEMED was added last. As soon as the TEMED was added, the gels were poured into tubes which had been previously soaked in Photoflow, washed, and dried in a hot air oven. The gels were overlaid with distilled water while the following stacking gel solutions were prepared:

	amount used (mL)
30% acrylamide, 0.8% bis acrylamide	1.0
0.25M Tris-Cl pH 6.8	5.0
ammonium persulfate 1.5%	1.0
distilled water	3.0
TEMED 100 %	0.010

Immediately following the addition of TEMED, the stacking gel solution was poured on top of the separating gel. The gels were then overlayed with distilled water and allowed to polymerize overnight.

METHODS

Synthesis of S-Mercuric N-dansylcysteine. (SMNDC)

SMNDC was synthesized according to the method of Leavis and Lehrer (103). Ten mL of a saturated solution of didansylcysteine (5×10^{-3}M) was stirred overnight with a 10 fold excess of dithiothreitol in 50mM Tris acetate buffer, pH 8.0. The beaker was wrapped in aluminum foil to protect the solution from exposure to light.

The pH was adjusted to 3.0 with concentrated hydrochloric acid to prevent the reoxidation of the SH groups, and the mixture was applied to a column of Sephadex G-10 (1.5 x 40cm) and eluted with 1mM HCl. Five-mL fractions were collected and 10μL aliquots were assayed for the presence of sulfhydryl compounds using DTNB in Ellman assay buffer, and for for dansyl groups by measurement of the absorbance at 328nm. The concentration of SMNDC in the pooled SMNDC fractions was determined both with DTNB (56) and from the A_{328} using an extinction coefficient of 3980 $M^{-1}cm^{-1}$ (103). These values, which were in agreeement, were used to calculate the equivalent amount of mercuric chloride to be added to the solution, which was stored in a brown bottle at 4°C.

Preparation of N-methyl-7-Hydroxyquinolinium iodide (57)

7-hydroxyquinoline (0.1g) was dissolved in 20 mL of anhydrous tetrahydrofuran. To this was added 0.1mL of methyl iodide. The

solution was stored in the dark for 5 days whereupon crystals formed. These were collected and recrystallized from a minimal volume of hot methanol after treatment with activated charcoal. (m.p. 245°C; literature m.p. (57) (249-50°C).

Preparation of N-methyl-7-(dimethylcarbamoyloxy)quinolinium iodide (57)

7-hydroxyquinoline (0.5g) was dissolved in 1M sodium methoxide in methanol (4 mL). To this was added dimethylcarbamoyl chloride (0.5mL). After boiling in a water bath for 5 minutes, the mixture was cooled to room temperature and water was added (15 mL). The product was extracted with chloroform (2 portions of 20 mL). The chloroform extract was washed with 0.1M potassium hydroxide (20 mL) and water (20 mL) and then dried overnight over anhydrous sodium sulfate. The solution was filtered and evaporated to a brown oil (0.4g). To this was added methyl iodide (2 mL)in DMF (9 mL). The mixture was heated for 20 minutes on a boiling water bath and cooled. Upon the addition of ether a gummy white solid precipitated out. This solid was filtered and recrystallized using hot methanol and activated charcoal. (m.p. 196-198°C; literature m.p. (57) 197°C).

Synthesis of the affinity ligand (6-aminocaproyl-6'-aminocaproyl-p-aminophenyl)trimethylammonium (dicap-p-PTA) and the corresponding *meta* analogue (dicap-m-PTA)

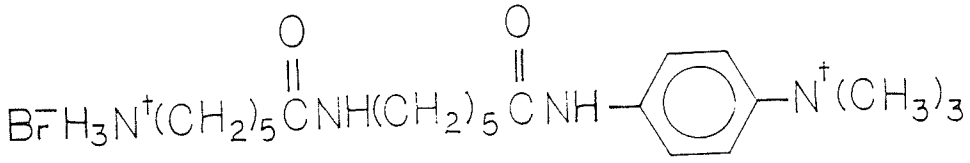
These compounds (Fig. 9) were synthesized by modification of methods described by Taylor *et al.* (112) and Dudai *et al.* (109).

Figure 9. Structure of the affinity ligands used in this study.

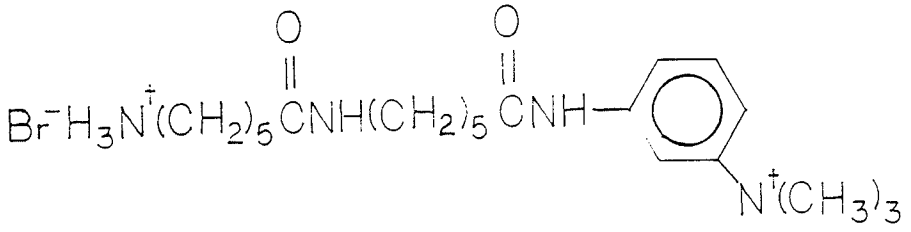
a: (6-aminocaproyl-6'-aminocaproyl-p-aminophenyl-trimethyl ammonium) bromide (for *E. electricus* AChE).

b: (6-aminocaproyl-6'-aminocaproyl-m-aminophenyl-trimethyl ammonium) bromide. (for *T. californica* AChE).

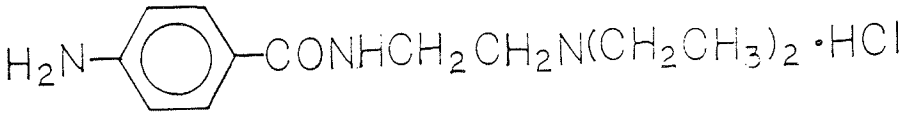
c: procainamide.HCl (for Human serum cholinesterase).



a



b



c

All solvents used in the syntheses were freshly dried and distilled. The 33% HBr in glacial acetic acid was freshly prepared before each use.

i) 6-carbobenzoxyaminocaproyl-m/p-N-N,dimethylphenylene diamine. (Cbz-cap-PDA)

N,N-dimethylphenylenediamine.HCl (10g of either *para-* or *meta-)* was dissolved in water (50 mL), and the pH adjusted to 9.0 with 3M NaOH. This mixture was then extracted with 3 portions of 60 mL each of anhydrous ether. The ether extract was dried overnight over anhydrous sodium sulfate. The dried extract was filtered, and evaporated to dryness. The brown oil solidified on cooling to yield the free base (8.5g).

Carbobenzoxy-6-aminocaproic (9.6g) acid was dissolved in dried ethyl acetate (120 mL). This mixture was cooled to -10^{o}C in an ice/salt bath. To the cooled mixture was added freshly distilled triethylamine (5.1 mL) and then isobutylchloroformate (5.1 mL). This mixture was stirred for 20 minutes, and filtered. To the filtered mixture was added the free base (5.0g) dissolved in cold ethyl acetate (50 mL) at a rate of 1 mL/min. A grey precipitate was formed. This mixture was left to stir for 3 hours at room temperature, and then left in the refrigerator overnight. The compound was filtered and washed with 10 mL portions each of cold water, cold ethyl acetate, and petroleum ether respectively. The white solid was recrystallized from hot methanol and activated charcoal, or methanol/ether.

ii) 6-aminocaproyl-p/m-N,N-dimethylphenylenediamine (cap-PDA)

Freshly prepared 33% HBr/glacial acetic acid (75 mL) was added to Cbz-6-aminocaproyl-p/m-N,N-phenylene diamine (6.6g) which had been dissolved in glacial acetic acid (30 mL), and stirred for one hour at room temperature. This mixture was added all at once to anhydrous ether (200 mL), and the gummy solid washed exhaustively with anhydrous ether until a white powdery material was obtained. The solid was precipitated from hot methanol and anhydrous ether and the hygroscopic solid was used directly in the next step.

iii) 6-carbobenzoxyaminocaproyl-6'-aminocaproyl-p/m-N,N-phenylene diamine. (Cbz-dicap-PDA)

Cbz-6-aminocaproic acid (3.3g) was dissolved in ethyl acetate (40 mL) and cooled to -10°C in an ice/salt bath with stirring. To this was added, in order, freshly distilled triethylamine (1.8 mL) and isobutyl-chloroformate (1.8 mL). This mixture was left to stir for 30 minutes and then filtered. To this filtered mixture was added cap-PDA (5.0g) dissolved in ethyl acetate (30-40 mL), and triethylamine (3.6 mL). This mixture was left to stir for 2 hours at room temperature, and refrigerated overnight. The solid was filtered and washed with 10 mL portions each of cold water,cold ethyl acetate, and petroleum ether. The purplish solid was precipitated from methanol and anhydrous ether.

iv) 6-aminocaproyl-6'-aminocaproyl-p/m-aminophenyl trimethyl ammonium (Cbz-dicap-PTA)

Cbz-dicap-m/p-PDA (3.0g) was dissolved in dimethylformamide (4.0 mL) methyl iodide (4.0 mL) and placed in a large screw cap test tube. The mixture was heated to 100°C in a boiling water bath for 40-60 minutes. After heating, the mixture was cooled to 0°C and cold ethyl acetate (0.5 mL)was added. An oil was formed and settled to the bottom of the test tube. The oil was dissolved in hot absolute ethanol and crystals were observed on cooling. Occasionally no oil layer was observed, in which case more cold ethyl acetate was added. If there was still no oil layer observed, the entire contents was added to anhydrous ether (100 mL), and the solid was washed exhaustively with anhydrous ether.

vi) 6-aminocaproyl-6'-aminocaproyl-p/m-aminophenyl trimethyl ammonium (dicap-m/p-PTA)

Cbz-dicap-m/p-PTA (0.5g) was dissolved in glacial acetic acid (5 mL). To this was added freshly prepared 33%HBr in glacial acetic acid (15 mL). This was allowed to react for 45-60 minutes, and the entire contents of the flask was added all at once to anhydrous ether (200 mL). This gummy solid was washed exhaustively with anhydrous ether. The resulting powder was very hygroscopic, but the compound could be precipitated out from dried methanol and anhydrous ether. The *p*-analogue eventually cystallized (m. p. 179°; literature m.p. 179-180° (77), but the *m*-analogue could not be crystallized.

Coupling of dicap-p/m-PTA to Sepharose 4B (113,114)

Packed Sepharose 4B (200 mL)was washed with 2L of cold water. To this was added cyanogen bromide (1.8g) either in solid form or dissolved in a minimal volume of acetonitrile. The pH was maintained at 11.0 +/- 0.5 by the addition of 3M NaOH, and the temperature was maintained at 15°C +/- 2°C using ice. After 20 minutes (or until the pH was no longer changing) the gel was washed with cold 0.5 M sodium bicarbonate (pH 9.8). The gel was then quickly transferred to a beaker containing dicap-PTA (0.4 millimole) dissolved in 0.5M sodium bicarbonate, pH 9.8 (200 mL). This was left to stir overnight and then washed with cold 0.5M sodium bicarbonate buffer, pH 9.8 (1 L), cold water (2L) and TPB (m-dicap-PTA) (2 L) or EQB (p-dicap-PTA) (2 L).

Coupling of the Procainamide to CH-Sepharose.(115)

The procainamide was used directly as received from Sigma Chemicals. The coupling procedure used was the one recommended and published by Pharmacia Fine Chemicals, and will be described here.

CH-Sepharose (15g) was swollen in 0.5M NaCl solution for one week. The swollen gel was washed with 200 mL/g of both 0.5M NaCl and cold water respectively, on a coarse sintered glass filter. The washed gel was transfered to a beaker and the pH adjusted to 4.5 such that the liquid to gel ratio is 2:1. To this was added the coupling agent EDAC (3.46g) dissolved in a minimal amount of water. The solution was added dropwise over a period of 30 min. while maintaining the pH at

5.0 with 1M HCl. After maintaining the pH at 5.0 for 13 hours, the gel was stored in the fridge overnight. The next day the pH of the gel was found to be 5.0 and was subsequently washed with several washes of 0.02M potassium phosphate/1M NaCl buffer, pH 5.0. The gel was washed with distilled water and then equilibrated with 0.02 M potassium phosphate, pH 7.0.

Isolation and Purification of Acetylcholinesterase from *Torpedo californica*

Frozen tissue (50-100g) was cut into small pieces and ground up in a Virtis homogenizer with Torpedo Preparative Buffer (1mL/g wet tissue). The homogenate was centrifuged for one hour at 16,000 rpm. The supernatant was decanted and the pellet was resuspended in TPB (1mL/g wet tissue). The resuspended homogenate was heated to 37°C and to this was added 1 μL/mL of Trypsin which was 5mg/mL in concentration. The mixture was stirred for 7-8 minutes, then 1μL/mL of Soybean Trypsin Inhibitor was added at a concentration of 10 mg/mL. The mixture was cooled rapidly to 15°C in an ice/salt bath, and centrifuged for 1 hour at 16,000 rpm.

The supernatant was decanted and loaded onto the dicap-m-PTA affinity column and the column was washed with TPB until the absorbance at 280 nm was less than or equal to 0.05 absorbance units (usually 15-20 column volumes was sufficient). The AChE was eluted from the column with 0.1M decamethonium bromide, until the A_{280} was less than or equal to 0.05 abosorbance units. The decamethonium bromide was removed from the protein using a carboxymethyl-Sephadex column. Yield was 5000-8000

units of enzyme as measured using the Ellman Assay.

Isolation and Purification of Acetylcholinesterase from *Electrophorus electricus*

The enzyme was used directly as supplied by Sigma Chemicals. 10,000 active units were dissolved in 5 mL of cold EQB loaded onto the dicap-p-PTA affinity column. The column was washed with EQB until the absorbance was less than or equal to 0.05 absorbance units at 280 nm. The protein was eluted with 0.1M decamethonium bromide, and then passed through a carboxymethyl-Sephadex column to remove the decamethonium bromide.

Isolation and purification of Human serum cholinesterase.

Outdated human plasma (10 L) was dialyzed against 25 litres of 0.02M sodium acetate/1mM EDTA pH 4.0 at 4°C. The buffer was changed at least twice. This takes 2-3 days. During this time a white "junk" protein settled to the bottom of the dialysis tubing. The dialysis tubing was punctured or slit with a razor blade just above the junk protein and the clarified plasma collected in 4L beakers.

DEAE cellulose (1000 g) was de-fined with 0.02M sodium acetate 1mM EDTA pH 4.0 and 400 grams added to each of the 4L beakers. The plasma and DEAE cellulose was stirred for 45-60 minutes, or until the activity was less than or equal to 500 units. The cellulose containing the cholinesterase was allowed to settle and the plasma decanted. The loaded resin was poured into a 1L coarse sintered glass filter, and

washed until the eluent was as clean looking as possible.

The washed slurry was then poured into a one-litre Pharmacia column and packed down with a pressure head of the the same 0.02M sodium acetate 1mM EDTA buffer (pH 4.0). After packing the loaded resin, the column was washed until the A_{280} was less than or equal to 0.05 abosrbance units. The column was eluted with a 0.2M sodium chloride gradient in the same 0.02M sodium acetate buffer.

The active protein was collected, pooled and diluted with 0.02M potassium phosphate buffer 1mM EDTA pH 7.0 until the ionic strength was equal to or slightly greater than that of the same 0.02M potasssium phosphate buffer. DEAE cellulose (750g) was de-fined with 0.02M potassium phosphate buffer, pH 7.0, and added to the pooled protein. The mixture was stirred for 45-60 minutes or until the activity was less than or equal to 500 units. The loaded resin was allowed to settle out, and the supernatant decanted. The loaded resin was poured into a 0.5L column and packed with a pressure head. Once the column was packed it was washed until the A_{280} was less than or equal to 0.05 absorbance units and eluted with a 500 ml 1M sodium chloride gradient in 0.02M potassium phosphate 1mM EDTA pH 7.0 to obtain the enzyme.

The procainamide affinity column was washed with 0.02M potassium phosphate buffer to equilibrate the column as well as to eliminate any excess procainaminde which would destroy the enzyme. The partially-purified enyzme was diluted such that the ionic strength was equal to that of the buffer, and then loaded onto the column. The column was washed until the A_{280} was less than or equal to 0.05 absorbance units, and eluted with a 500mL 0.8M sodium chloride gradient in the same 0.02M potasium phosphate buffer 1mM EDTA pH 7.0. The protein was usually

very clean and very pure. If not, then another DEAE celluose column was run and the column eluted with a 0.25M choline gradient in 0.02M potassium phosphate buffer, pH 7.0. The choline was then dialyzed out using an Amicon dialysis apparatus.

Reduction of Lysozyme

The procedure used was as described by Jori *et al.* (110). Lysozyme (30 mg) was dissolved in a 5%(aq) solution of β-mercaptoethanol to a final concentration of 0.05 mg/mL, pH 8. This was left to stir in a nitrogen atomosphere for approximately 24 hours. The pH was adjusted to 3 and the mixture separated on a G-25 Sephadex column using 0.2M acetic acid as the eluent. The solution was concentrated to a small volume using a Millipore CX-10 apparatus.

Enzyme assays

M7C assay (58)

A standard curve was run using M7H as a standard. A stock solution of 1.64mM M7H was prepared by dissolving 4.7mg in 10 mL of water. The working M7H standard was made by a 1000 fold dilution of the stock solution. To 2 mL of 1mM Tris-Cl pH 7.4 in disposable polystyrene cuvettes were added 5μL aliquots of M7H, and the fluorescence intensity measured. The measurements were performed on a Perkin-Elmer MPF-44 spectrofluorimeter at λ_{ex} 405nm and λ_{em} 505nm.

A stock solution of 2mM M7C was prepared by dissolving M7C (7.2 mg)

in water (10 mL). The assay was run by adding 5μL of M7C stock to 2mL of 1mM Tris-Cl pH 7.4, then adding between 50-100μL of enzyme mixture to the solution and monitoring the burst titration due to the carbamoylation of the enzyme and the consequent release of M7H. Enzyme concentration was determined from the amplitude of the carbamoylation phase by comparison with the M7H standards.

Modified Ellman Assay (56)

Commercial DTNB was recrystallized as the sodium salt (116). DTNB (1.0g) was dissolved in a solution of 0.425 g of $NaHCO_3$ in 5 mL of distilled water. This was heated gently (40-50°C) and stirred until the foaminess was gone, and the solution became transparent. This mixture was then filtered, and 45 mL of N-propanol added. The solution was left to sit at 0-5°C overnight. The crystals were collected and washed with a small portion of cold n-propanol.

Ellman assay buffer (3.0mL), stock DTNB (0.1 mL), stock acetylthiocholine (20μL) and enzyme (5μL) were mixed together and the rate of release of thionitrobenzoate was monitored at λ_{max} 412nm using a Hewlett-Packard HP8452A diode-array spectrophotometer.

Benzoylcholine Assay (111)

Benzoylcholine assay buffer (2.9 mL) was placed into a cuvette along with benzoylcholine (0.1ml, A_{247}=1.875). To this was added 5-30μL of enzyme (human serum cholinesterase) depending upon the volume and activity. BChE activity was determined from the decrease in A_{247}

with time.

Sulfhydryl group titrations

Titrations of protein thiol groups with DTNB were carried out using protein solutions having an apparent thiol concentrations in the range of 2×10^{-5} to 1×10^{-4}M, and done as outlined by the Ellman assay. The sulfhydryl group concentration was calculated using a molar extinction coefficient of 1.36×10^{4} (103).

Titrations of protein thiol groups with SMNDC were carried out using solutions of apparent thiol concentration in the range of 0.1-10μM. To 3.0 mL samples of protein solutions were added 5μL aliquots of SMNDC solution such that the final concentration of the reagent covered the range of 0.20-2.0 times the thiol concentration. Between additions and following an appropriate incubation period in the dark, the fluorescence intensity was measured at 495nm (excitation at 330 nm) using a Perkin-Elmer MPF-44 spectrofluorimeter. The buffers used were 1-10mM Tris-Cl pH 7.5 with or without either 0.1M NaCl or 1×10^{-4}M $ZnSO_4$. Unless otherwise stated all measurements were carried out at 25°C.

In some experiments the effects of ligands ($ZnSO_4$, $MgSO_4$, d-tubocurarine, and gallamine) on SMNDC-AChE fluorescence was studied using enzyme which had been labeled under conditions of low ionic strength as previously outlined. An aliquot of each ligand was added to respective samples of SMNDC-labeled enzyme solution and the fluorescence intensity at 495nm was measured until no further change was observed.

SDS-PAGE

Sample preparations

The sample buffer consisted of 0.2 mg bromophenol blue, 66 mg DTT, 5 mL glycerol, 4 mL water, and 1 mL of 20% SDS. Protein solutions were made as follows: 0.6 mL sample buffer, 0.6 mL 1.5 M Tris-HCl buffer pH 8.8 and 1.2 mL protein sample. The protein samples were boiled for 10 minutes, cooled and applied to the gels. In the case of SMNDC-labeled proteins, DTT was omitted in order to preserve the mercaptide linkage between protein and label. The amounts of SMNDC-labeled proteins used were adjusted so that the final concentration of SMNDC was the same for all proteins. Duplicate sets of gels were run and one set was stained with Coomasie Brilliant Blue and the other inspected for fluorescence under ultraviolet light.

Gel Filtration Studies

Gel chromatography of protein and labeled protein mixtures were carried out on a 40 or 70 x 0.9 cm column of Sephadex G-25, using 10mM Tris/0.1MNaCl pH 7.5 as eluent at a flow rate of 12 mL/h. The eluted fractions were monitored for absorbance at 280 nm, and for dansyl fluorescence as appropriate.

RESULTS

Synthesis of affinity ligands

The synthesis of m- and p-dicap-PTA was as described by Mutus (107). Dicap-p-PTA was found to be a beige to white hygroscopic powder which melted at 179°C and showed a characteristic U.V. absorption band, λ_{max} 243 nm. Both properties were in accord with those reported in the literature (77). Dicap-m-PTA was isolated as an orange-brown compound which also had λ_{max} at 243nm. An accurate melting point could not be obtained as the compound was extremely hygroscopic. Both compounds were coupled to Sepharose 4B by the cyanogen bromide method.

The amount of ligand actually coupled to the gel was difficult to control and numerous batches of affinity gel had to be prepared and tested in order to obtain a working affinity column, a time consuming and expensive process. The level of ligand substitution was critical in that highly substituted gels either bound the enzyme irreversibly or the protein that eluted from the column was devoid of AChE activity. Too little ligand bound to the gel leads to non-retention of the enzyme. Best results were obtained for both *T. californica* and *E. electricus* AChE using columns containing approximately 0.4 micromoles of ligand/mL of gel (estimated by spectrophotometric assay of the washings obtained after coupling). On the positive side, a working affinity column remains functional for several months.

Purification of 11s acetylcholinesterase from *Torpedo californica* and *Electrophorus electricus*

Affinity chromatography of 11s AChE from *E. electricus* and *T. californica* was carried out very efficiently using Sepharose 4B-linked dicap-p-PTA and dicap-m-PTA, respectively.

Commercial eel AChE (Sigma type VI-S, 10,000 units, specific activity about 400 U/mg) were completely adsorbed on the column and subsequently eluted with DMB as a single peak. There were 8500 units of activity recovered after removal of DMB, with specific activities in the range 6-8000 U/mg. Highly purified AChE is reported to have a specific activity of 10,000 U/mg (129).

Torpedo AChE was isolated from trypsin treated electroplax tissue in 85% yield. The specific activity was about 6500 U/mg, in excellent agreement with the reported value of 6500-7000 U/mg (77). The enzyme thus appeared to be of very high purity. SDS-polyacrylamide gel electrophoresis showed only those bands expected for AChE (not shown, see Ref. 82).

Purification of Human serum cholinesterase (BChE)

BChE was purified using a combination of ion exchange and affinity chromatography. BChE (4720 units at a concentration of 1.18 U/mL) was obtained after exhaustive dialysis of about 4L of plasma. After batch-loading onto DEAE-cellulose at pH 4.0 followed by NaCl gradient elution, the enzyme was obtained in a narrow band marginally ahead of a contaminant peak (Fig.10A). 4261 units of activity (90%) were

recovered, with a specific activity of 14.1 U/A_{280}. A second ion-exchange chromatography was sometimes employed, with recovery of 3927 units (92%), specific activity 14.3 U/A_{280} (Fig. 10B). Affinity chromatography on procainamide-CH-Sephadex followed by elution with 0.02M NaCl yielded 3350 units (85%) of BChE with specific activity $168U/A_{280}$ (Fig. 10C). Based on a specific activity standard of $200U/A_{280}$ (Lockridge, personal communication), the enzyme was judged to be 84% pure. SDS-PAGE indicated the presence of a monomer of M. W. 85 kDa and a dimer of 170 kDa, with only minor contamination (Fig. 11).

Synthesis and properties of SMNDC

SMNDC was synthesized by the method of Leavis and Lehrer (102). Didansyl cysteine was reduced using a ten fold excess of dithiothreitol at pH 8.0, followed by removal of DTT by gel chromatography on Sephadex G-10. The fractions containing dansylcysteine were used in the next step only if the concentration of Dns-cys determined spectrophotometrically (λ_{max} 328 nm, ε_{max} 3980 $M^{-1}cm^{-1}$) was in agreement with the SH content measured by reaction with DTNB. These numbers served to establish the amount of mercuric chloride to be added to produce a stoichiometric amount of SMNDC. Precise estimation of this quantity is crucial to the successful utilization of SMNDC as a probe of protein-bound thiol groups The reaction of mercuric chloride with dansylcysteine proceeds *via* the initial formation of a disubstituted mercury mercaptide; further addition of the mercuric salt yields the desired product:

$$2R\text{-}SH + Hg^{2+} \longrightarrow R\text{-}S\text{-}Hg\text{-}S\text{-}R \xrightarrow{Hg^{2+}} 2RS\text{-}Hg^{+}$$

Figure 10. Purification of BChE.

DEAE cellulose chromatography at: A. pH 4.0
B. pH 7.0

followed by C. procainamide affinity chromatography.

(---) A_{280}.
(——) Human serum cholinesterase activity.

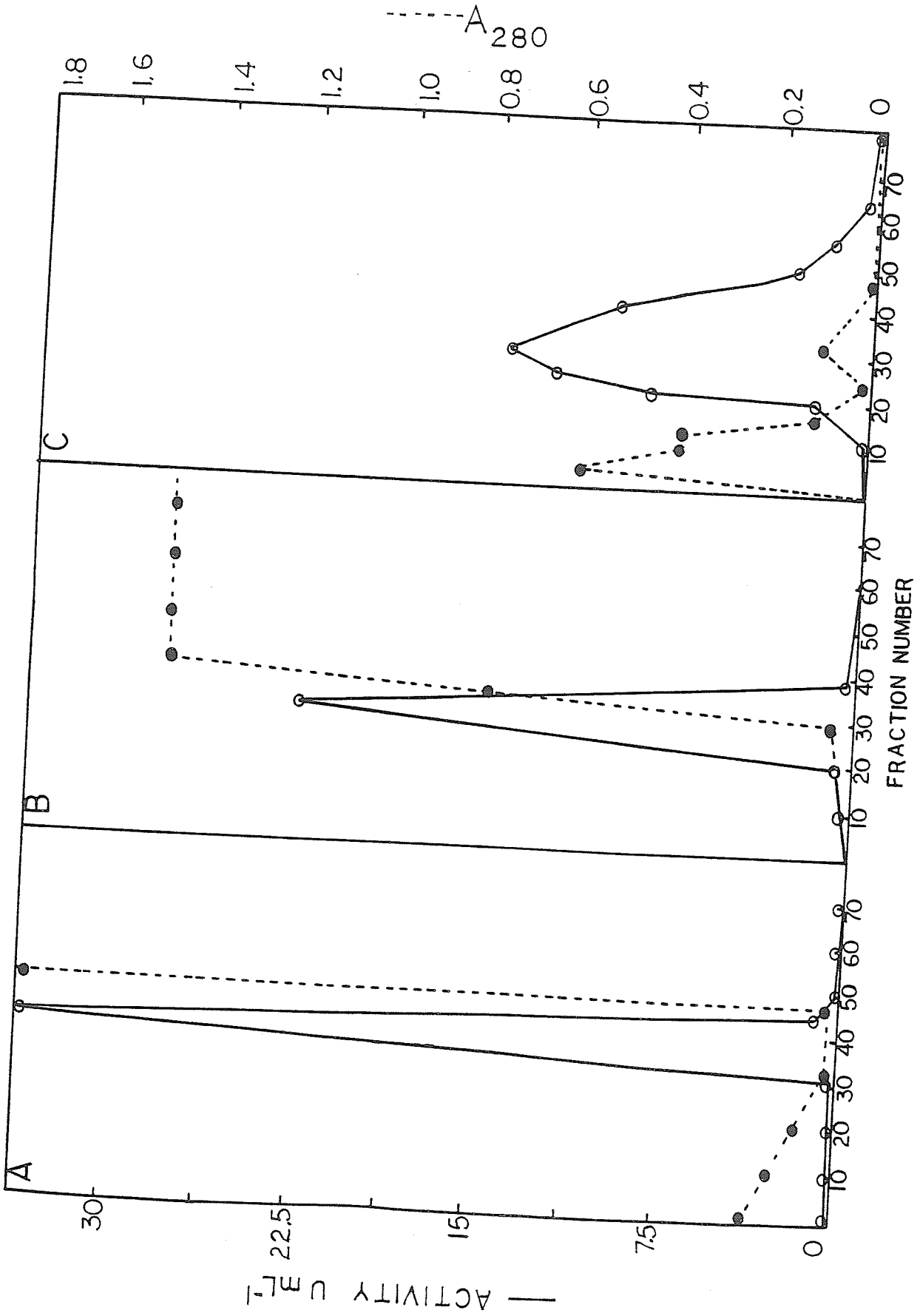

A280
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A
B
C
30
22.5
15
7.5
0
10
20
30
40
50
60
70
FRACTION NUMBER
ACTIVITY U mL-1

Figure 11. Purification of BChE.

SDS-PAGE (Coomasie Blue stain) of:

A. Molecular weight marker proteins
B. Purified Butyrylcholinesterase.

A B

If too little mercuric chloride is added, even if only by a small amount, the solution may contain residual amounts of the disubstituted mercaptide. This compound competes with SMNDC for reaction with protein-bound sulfhydryl groups with concomitant release of 1 mole of dansyl cysteine for each dansyl group bound to the protein:

$$R\text{-}S\text{-}Hg\text{-}S\text{-}R + P\text{-}SH \longrightarrow R\text{-}S\text{-}Hg\text{-}S\text{-}P + R\text{-}SH$$

The fluorescence intensity of dansyl cysteine is about three times that of SMNDC. For those proteins which produce only a small enhancement of SMNDC fluorescence after reaction with the reagent, the presence of any free dansylcysteine formed as described above can generate misleading results and inaccurate estimates of the thiol content.

On the other hand, addition of excess mercuric chloride often resulted in the formation of a precipitate of unknown nature, possibly a polymeric mercury-SMNDC complex (S. Loeb, personal communication). This compound could be re-dissolved by addition of a few drops of 1M Tris buffer yielding a solution whose spectral and fluorescence properties appeared identical to those of genuine SMNDC. However, such solutions invariably yielded inconsistent results and spectral abnormalities when used to examine protein thiol groups. For these reasons, the SMNDC preparations were subject to careful scrutiny to ensure their spectral and fluorescence properties matched those given by Leavis and Lehrer (102). Solutions of SMNDC were generally prepared fresh every 2-3 days.

Characteristics of the reaction of SMNDC with proteins

a) Studies with model proteins

In order to establish the characteristics and criteria for covalent binding of SMNDC to proteins, four proteins were chosen to serve as models. These were BSA, S-carboxymethyl-BSA, lysozyme and reduced lysozyme. BSA as purchased from the suppliers normally contains between 0.6-0.8 SH groups/mol of protein as measured by DTNB titrations. S-carboxymethyl-BSA contains less than 0.02 moles of DTNB-titratable SH groups per mole of protein. Native lysozyme has no free SH groups, but one of the two disulfide linkages can be easily reduced to give an active species containing two SH groups per mole of protein (109).

Addition of stoichiometric amounts of SMNDC to BSA and S-carboxymethyl-BSA resulted in an immediate, large increase in fluorescence accompanied by a blue shift in the SMNDC fluorescence emission maximum from 535nm to 495 nm. (Fig. 12A). Native lysozyme does not interact strongly with SMNDC, whereas the reduced form of lysozyme (2.08 SH groups per mole protein by DTNB) shows an increase in the fluorescence also accompanied by a blue shift from 535 nm to 495 nm. In the case of reduced lysozyme, the increase in fluorescence required 4-5 hours and the final intensity is one half that of the fluorescence intensity of both BSA and S-carboxymethyl-BSA (Fig. 12A). Figure 12B shows the titration curves obtained with BSA or S-carboxymethyl-BSA, and reduced lysozyme. This indicates that the binding is tight in all cases. However, the binding of the probe to BSA or S-carboxymethyl-BSA is non-covalent since the probe and the

Figure 12. The interaction of SMNDC with BSA/carboxymethyl-BSA, and partially reduced lysozyme.

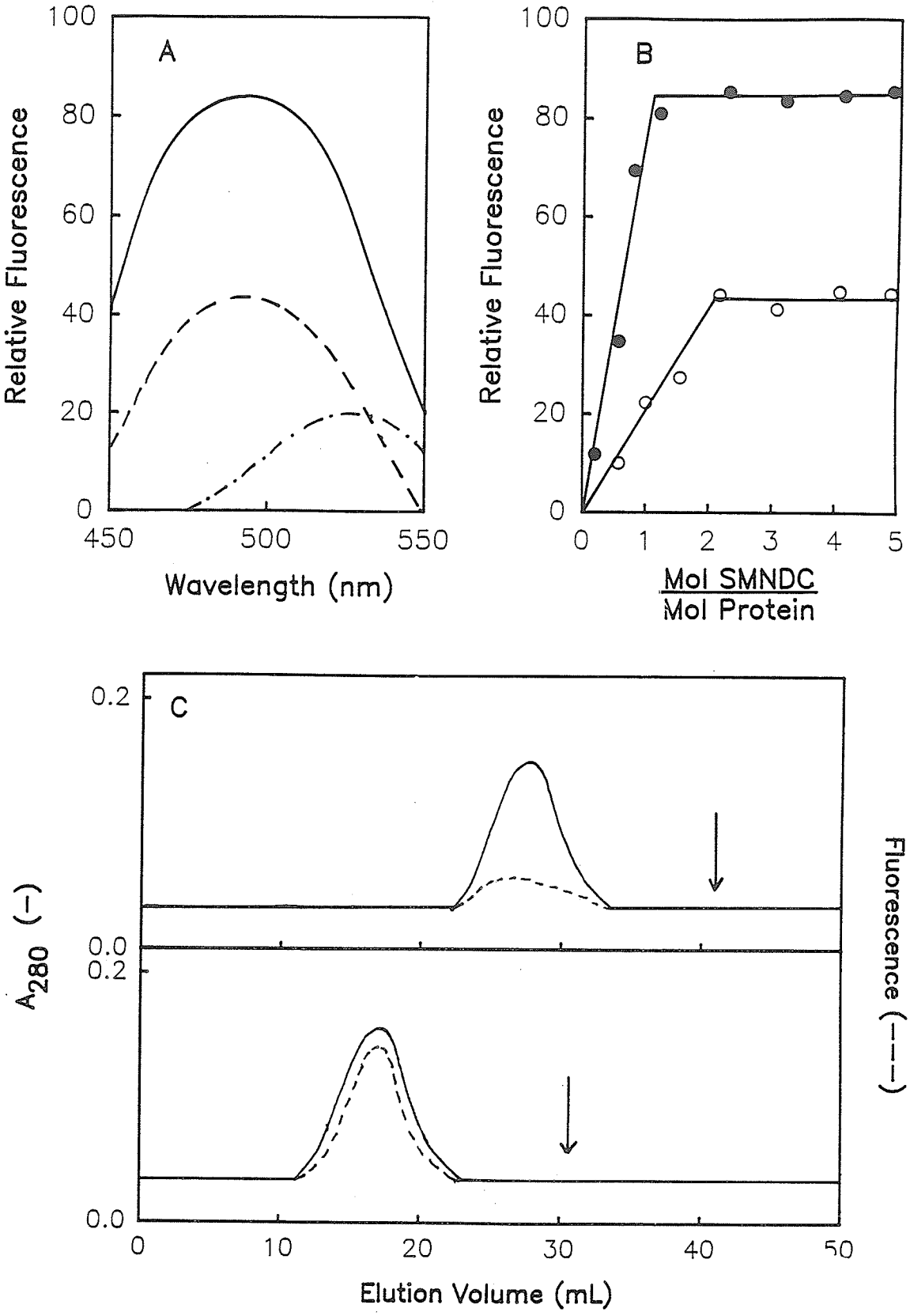
A. Fluorescence spectra of: 9.3μM SMNDC-BSA (or S-carboxymethyl-BSA). 1:1 complex (-); 4.5μM SMNDC-partially reduced lysozyme, 2:1 complex (---); 9.3μM SMNDC (-·-·).

All spectra were recorded in 1.0 mM Tris buffer, pH 7.5, λ_{ex}330 nm, λ_{em}495 nm.

B. Titration of 9.3μM BSA (or S-carboxymethyl-BSA) (●) and 4.5μM partially-reduced lysozyme (2.08 mol SH/mol protein) (○) with SMNDC.

C. Gel filtration of SMNDC protein complexes on Sephadex G- 25.

Upper trace: SMNDC-S-carboxymethyl-BSA, 70 x .90 cm column. Protein recovered was 90%, bound dansyl recovered was 10%. Lower trace: SMNDC-reduced lysozyme complex, 40 x 0.9 cm column. Recovery of the protein and fluorescence was greater than 90%. Buffer used was 10mM Tris/0.1M NaCl, pH 7.5. Arrows indicate the position of free SMNDC when chromatographed in the absence of protein.


A
Relative Fluorescence
100
80
60
40
20
0
450
500
550
Wavelength (nm)
B
Relative Fluorescence
100
80
60
40
20
0
0
1
2
3
4
5
Mol SMNDC
Mol Protein
C
0.2
0.0
0.2
0.0
A_{280} (—)
Fluorescence (– – –)
0
10
20
30
40
50
Elution Volume (mL)

protein could be separated by gel filtration on a Sephadex G-25 column, as shown in Figure 12C. No peak for free SMNDC was observed, presumably because of the tight binding of the probe to the protein which results in slow dissociation and the SMNDC is lost in the baseline. However, analysis of the protein peak for SMNDC showed that greater than 90% of the original label was lost after gel chromatography. In addition to this, the addition of DTT to the reagent-protein mixture to reduce any SMNDC-protein mercaptide linkages, which should result in loss of fluorescence, had no effect upon the fluorescence spectrum in either case. Thus, the thiol group of BSA is not accessible to the SMNDC probe, due tight noncovalent binding of the probe to another site which is also present in the S-carboxymethyl-BSA protein.

SMNDC and reduced lysozyme co-chromatograph on Sephadex G-25 as is observed in Figure 12C. In this case, the addition of an excess of DTT abolishes the fluorescence by breaking the mercaptide-protein bond. SMNDC thus binds covalently to reduced lysozyme.

Other examples of covalent bonding of SMNDC with proteins are shown in Table I. Of the few examples of SMNDC-protein reactions found in the literature, only two do not have a finite reaction time. This feature, as well as the loss of fluorescence following addition of reducing agent, and co-chromatography (or electrophoresis) of probe and protein, would appear to be provide strong evidence for the covalent reaction of SMNDC with thiol-containing proteins.

b) Reaction of SMNDC with AChE

The reaction of SMNDC with affinity purified 11s *T. californica*

TABLE I. Time required to reach maximum fluorescence in the reaction of Dns-cys-SHg^+ with thiol- containing proteins.

Protein	Reaction time (min)	Reference
Troponin C	Instantaneous	Leavis & Lehrer (1974)
Tropomyosin	Instantaneous	Leavis & Lehrer (1974)
Actin	10	Leavis & Lehrer (1974)
Na^+/K^+-ATPase	45	Harris & Stahl (1976)
Ca^{2+}-ATPase	10	Ikemoto et al. (1978)
Galactosyltransferase	120	O'Keeffe et al. (1980)
Thiolase I	30	Izbicka-Dimitrijevic & Gilbert (1982)
Lysozyme (reduced)	300	This study
T. californica AChE	210	Mutus et al. (1983); this study
Human serum BChE	120	This study

AChE meets all of the above criteria for the covalent reaction of SMNDC with the protein. Stoichiometric amounts of SMNDC (4mol/mol enzyme) required 3.5 hours for complete reaction at 25°C (Fig. 13A). During this time, the characteristic spectral and fluorescence changes occur, which are subsequently abolished upon addition of an excess of DTT (Fig. 13B).

Figure 14 illustrates the titration of the enzyme with aliquots of SMNDC and very clearly shows the presence of 4 thiol groups/mol AChE under high or low ionic strength conditions. At high ionic strength (0.1M NaCl), the fluorescence is approximately 45% of that obtained under low ionic strength conditions. Titration in the presence of 1×10^{-4}M Zn^{2+} showed an apparent stoichiometry of SMNDC incorporation of 6 mol/mol enzyme. The final fluorescence intensity was 2.7 times that of the low ionic strength control.

AChE from *E. electricus* was completely unreactive towards SMNDC under all of the conditions used.

c) Reaction of SMNDC with BChE

Human serum cholinesterase also exhibits all of the characteristics of a covalent reaction with SMNDC, although the fluorescence enhancements are not as dramatic as those seen with the *Torpedo* enzyme and require a higher protein concentration in order to observe the reaction (Fig. 15).

Incremental addition of SMNDC to a known amount of enzyme generated a titration curve from which the SH content of BChE was determined to be 4 SH/mol under conditions of low ionic strength

Figure 13. Reaction of T. californica acetylcholinesterase with SMNDC.

A. Time course of the reaction. Change in fluorescence intensity at 495nm with time (λ_{ex}330nm). DTT (final concentration 1mM) was added after 3 hours, resulting in a total loss of fluorescence due to bound SMNDC.

B. Fluorescence emission spectrum of the solution from A immediately prior to addition of DTT. (-)
Spectrum of 0.175μM SMNDC is shown for comparison(---).

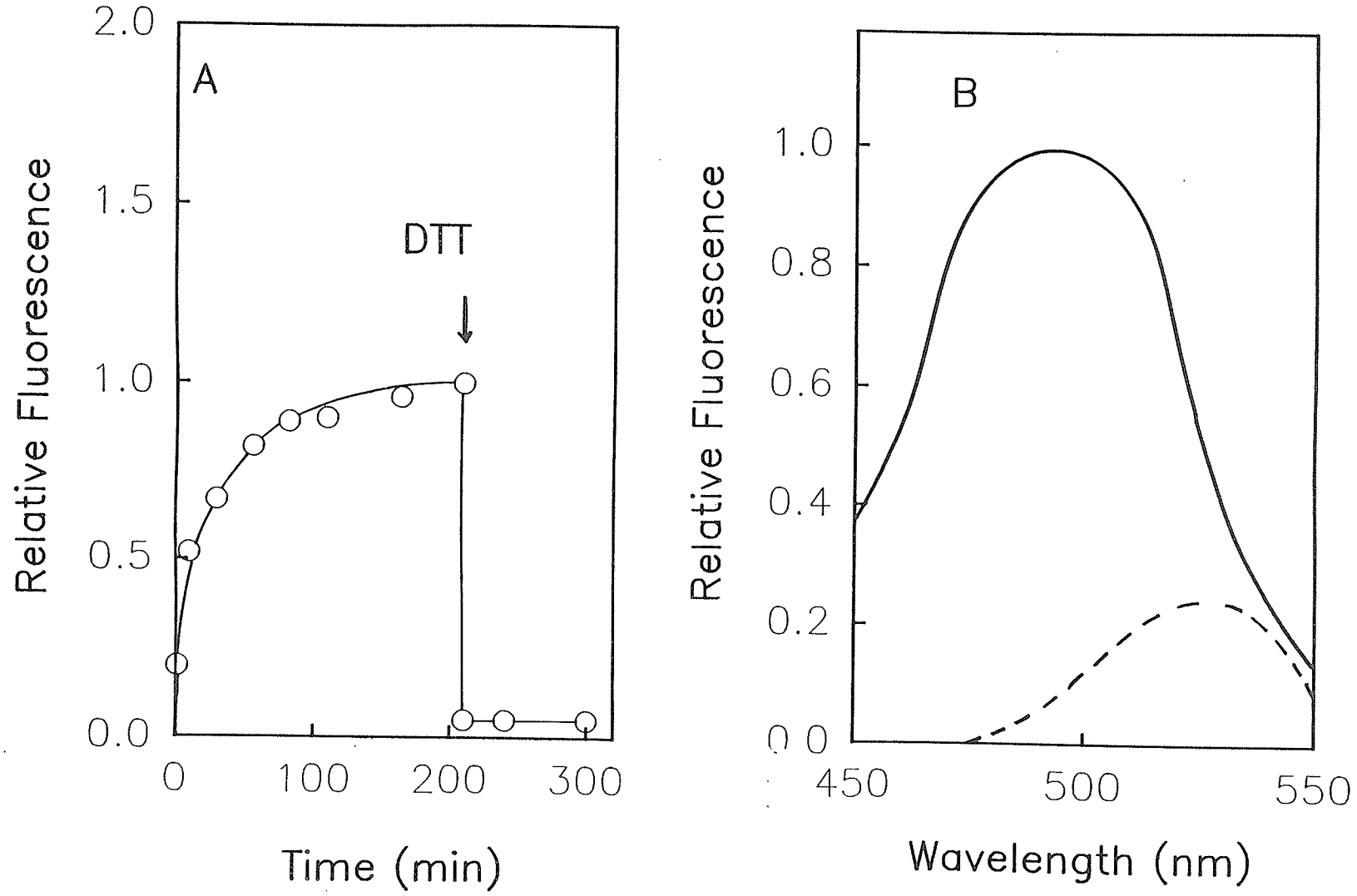

A
DTT
Relative Fluorescence
2.0
1.5
1.0
0.5
0.0
0
100
200
300
Time (min)
B
Relative Fluorescence
1.0
0.8
0.6
0.4
0.2
0 0
450
500
550
Wavelength (nm)

Figure 14. Stoichiometric titration of T. californica AChE (4.4×10^{-8}M) with SMNDC.

(●) in 1.0mM Tris, pH 7.5
(○) in 1.0mM Tris/0.1M NaCl, pH 7.5
(△) in 1.0mM Tris/10^{-4}M $ZnSO_4$, pH 7.5

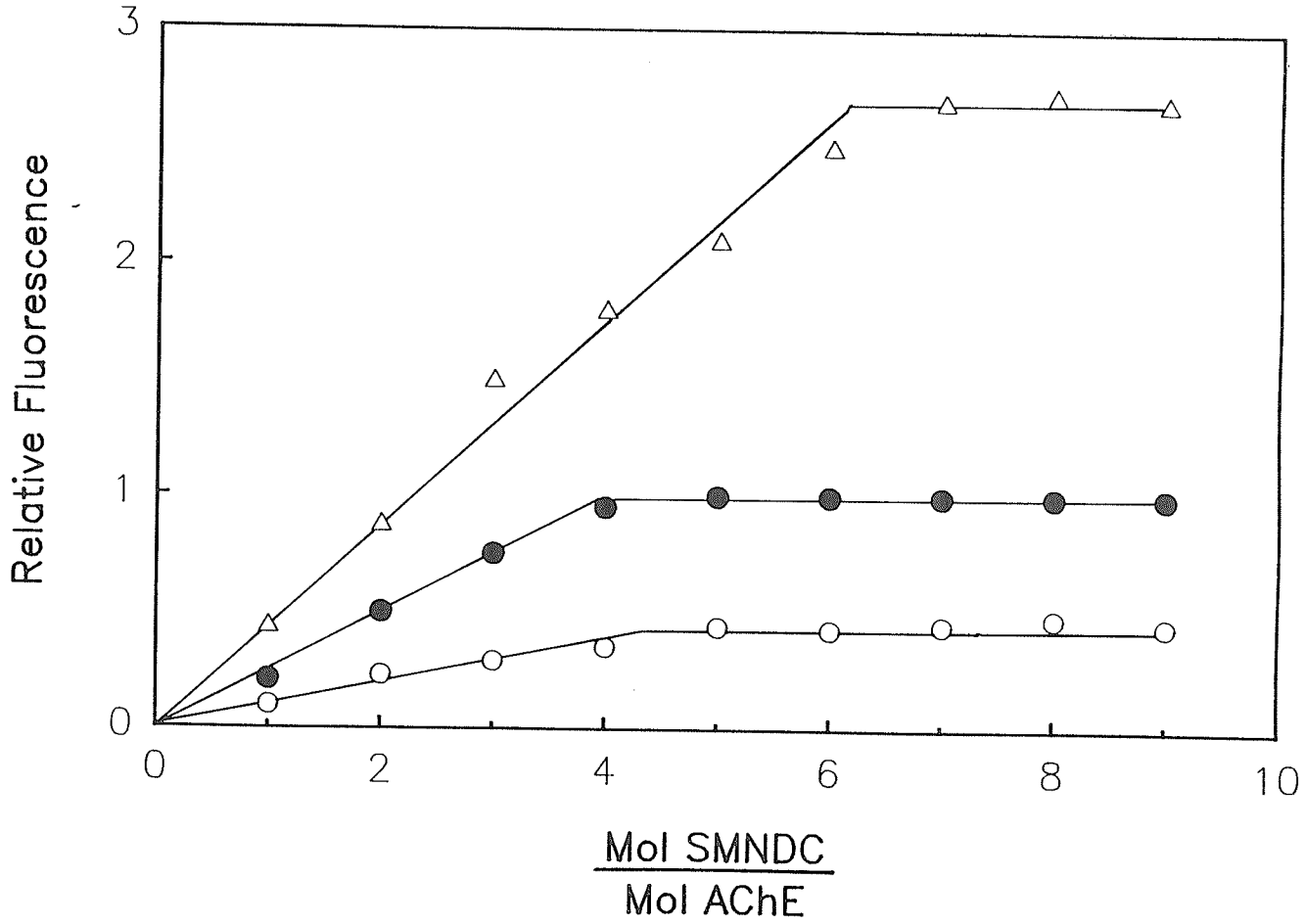
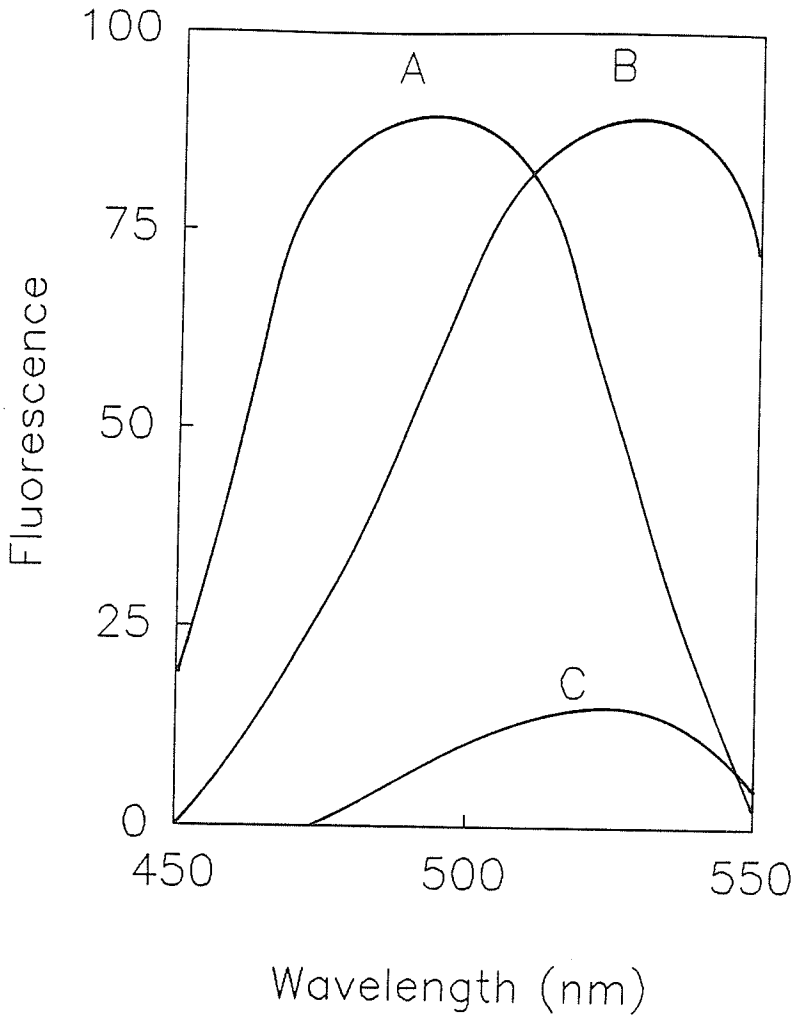

Relative Fluorescence
3
2
1
0
0
2
4
6
8
10
Mol SMNDC
Mol AChE

Figure 15. Fluorescence emission spectra.

A. SMNDC-labeled butyrylcholinesterase (6.3×10^{-7}M BChE; 2.52×10^{-6}M SMNDC).

B. The above solution after the addition of DTT to 1.0 mM OR a solution of dansylcysteine (2.52×10^{-6}M).

C. SMNDC (2.52×10^{-6}M).


100
75
50
25
0
Fluorescence
A
B
C
450
500
550
Wavelength (nm)

(Fig. 16). In the presence of Zn^{2+}, the fluorescence was enhanced approximately 2-fold but without any increase in the stoichiometry of SMNDC incorporation. A satisfactory titration could not be achieved in the presence of 0.1M NaCl owing to the low fluorescence intensity found in this medium.

d) SDS-polyacrylamide gel electrophoresis of SMNDC-labeled proteins

The covalent nature of the linkage between SMNDC and BChE can also be demonstrated using SDS-PAGE under non-reducing conditions (Fig. 17). BChE and the control (noncovalent) protein S-carboxymethyl-BSA were labelled with SMNDC such that the concentration of bound SMNDC was the same for each protein. Under these conditions, the fluorescence intensity of the SMNDC/S-carboxymethyl-BSA complex is about 8 times higher than that of SMNDC/BChE. After electrophoresis, the fluorescent bands of the labeled BChE were clearly visible under ultraviolet light at points corresponding to the protein bands run on parallel gels and stained wih Coomassie blue. However, the S-carboxymethyl-BSA-SMNDC complex after electrophoresis retains only traces of the label.

Reaction of BChE with DTNB

In a single experiment (owing to limited availability of enzyme), the reaction of BChE with the chromogenic thiol reagent DTNB was examined (Fig. 18). The estimated thiol content based on the measured A_{410} and an ε_{410} of 1.36×10^4 $M^{-1}cm^{-1}$ was in good agreement with that obtained in the SMNDC titration experiment.

Figure 16. Titration of butyrylcholinesterase with SMNDC.

Samples of enzyme (6.3×10^{-7}M) in 1.0mM Tris buffer, pH 7.5, were incubated with various amounts of SMNDC for 2 hours in the dark either in the absence (○) or presence (●) of 1.0×10^{-4}M $ZnSO_4$. Fluoresence intensity at 495 nm (λ_{ex} 330nm) was then recorded.

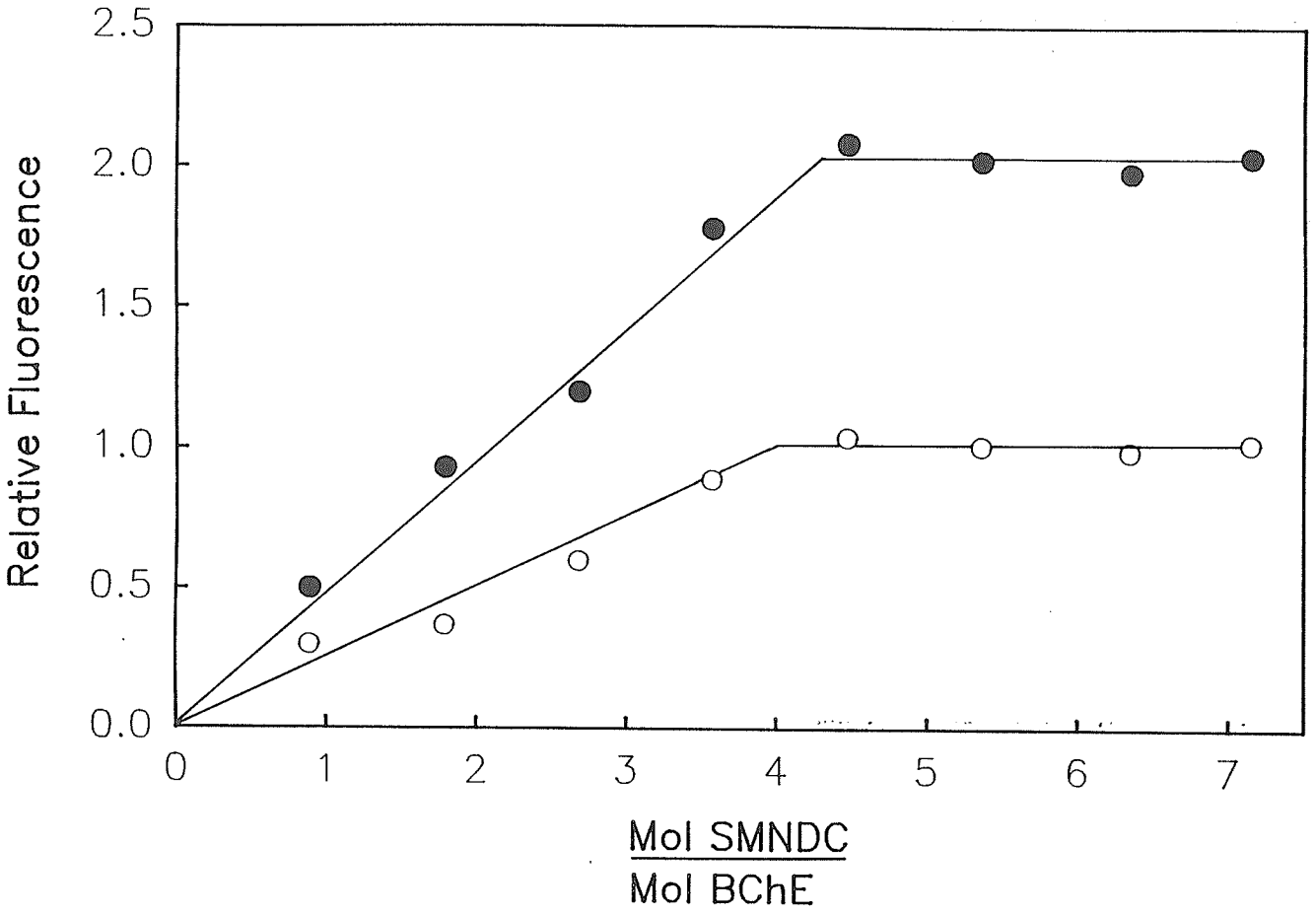

Relative Fluorescence
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
7
Mol SMNDC
Mol BChE

Figure 17. SDS-polyacrylamide gel electrophoresis under non-reducing conditions.

Proteins were labeled with SMNDC and duplicates of each were run on 10% gels such that each sample (except the standards) contained an equal amount of bound SMNDC. One of each of the gel samples was stained with Coomassie Blue while the other was examined under a UV light.

A. Molecular mass standards (myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin), stained with coomassie Blue.

B. BChE-SMNDC, 4 mol SMNDC/mol BChE; stained with Coomassie blue.

C. Same as lane B; visualized under UV light.

D. S-carboxymethyl-BSA-SMNDC, 1:1 complex, stained with Coomassie blue.

E. Same as lane D; visualized under UV light.

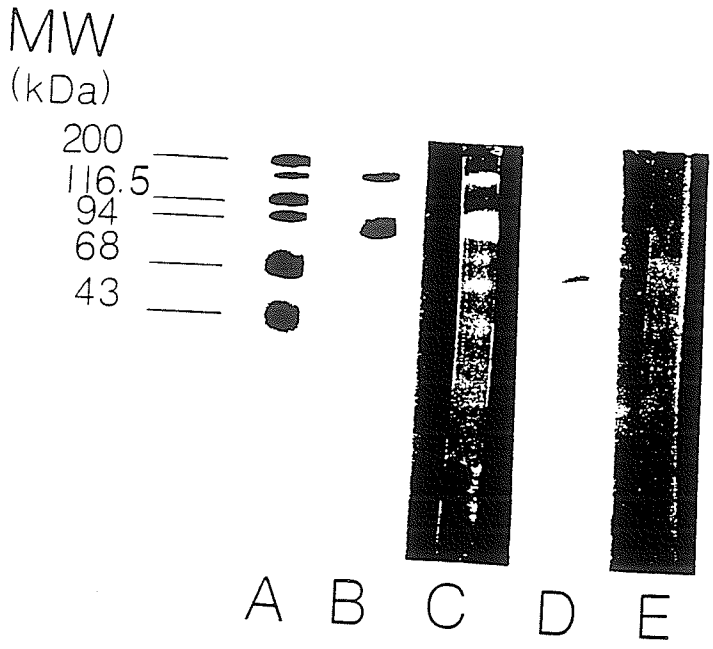

MW
(kDa)
200
116.5
94
68
43
A
B
C
D
E

Figure 18. Visible spectrum of a solution of BChE and DTNB.

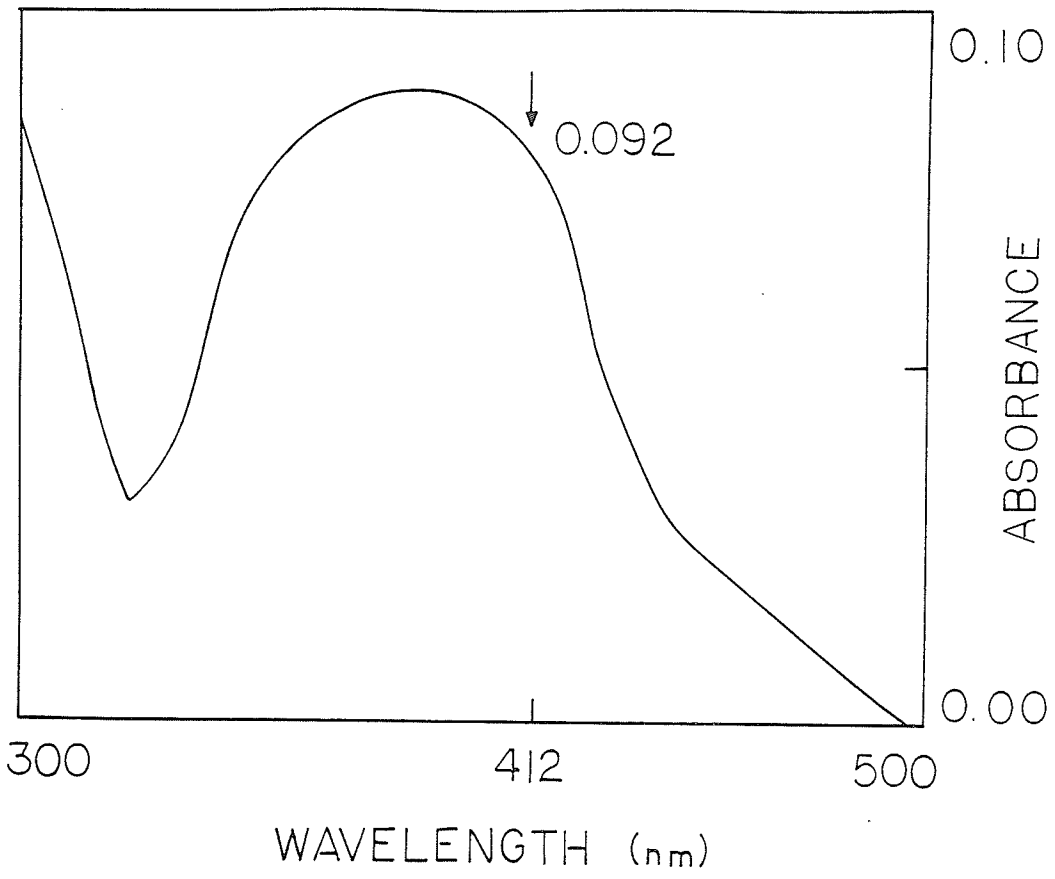
$A_{280} = 0.472$

[protein] $= 0.472/17,600$
$= 2.68 \times 10^{-5}$ M

[DTNB] $= 0.092/13,600$
$= 6.76 \times 10^{-6}$ M

number of SH groups: $2.68 \times 10^{-5} / 6.76 \times 10^{-6}$

$= 3.96$/mole protein.


0.10
0.092
ABSORBANCE
0.00
300
412
500
WAVELENGTH (nm)

SMNDC as a potential probe of AChE function

a) Effect of SMNDC labelling on enzyme activity

Torpedo AChE containing 4 moles of bound SMNDC per mole of enzyme retains more that 90% of the control activity towards the active-site carbamoylating agent M7C, and is as stable as the unmodified enzyme over a period of several hours (Fig. 19). It has been shown previously that acetylcholinesterase is inactivated by certain metal ions, most notably the Group IIB cations Zn^{2+}, Cd^{2+} and Hg^{2+} (86). Fig. 19 also demonstrates that SMNDC-labeled AChE retains this sensitivity towards inactivators, with the loss of activity following a time course identical to that found with unmodified enzyme. Both unlabeled and SMNDC-labeled BChE are also carbamoylated by M7C and, again, the activity is lost in the presence of Zn^{2+} (Fig. 20).

b) Effects of cations on SMNDC fluorescence and reactivity

The ability of Zn^{2+} to promote access to additional SMNDC-reactive groups on *T. californica* AChE along with an enhancement of bound dansyl fluorescence was further investigated by monitoring the changes in fluorescence of the labeled enzyme after the sequential addition of Zn^{2+} and extra SMNDC (Fig. 21). When Zn^{2+} (1×10^{-4} M) is added to a solution of labeled enzyme prepared in the absence of the cation (4 mol SMNDC/mol enzyme), a slow increase in the fluorescence is observed, reaching a value about twice that of the initial intensity after 3

Figure 19. Effects of SMNDC on AChE activity.

T. californica AChE (4.4×10^{-8}M) was incubated with SMNDC (1.75×10^{-7}) for 2.5 hours to effect labelling of the enzyme. The M7C carbamoylation aplitude was then measured at intervals for a further 3.5 hours followed by the addition of $ZnSO_4$ to a final concentration of 10^{-4}M.

A. (●) SMNDC-labelled enzyme.

B. (○) control enzyme in the absence of SMNDC.

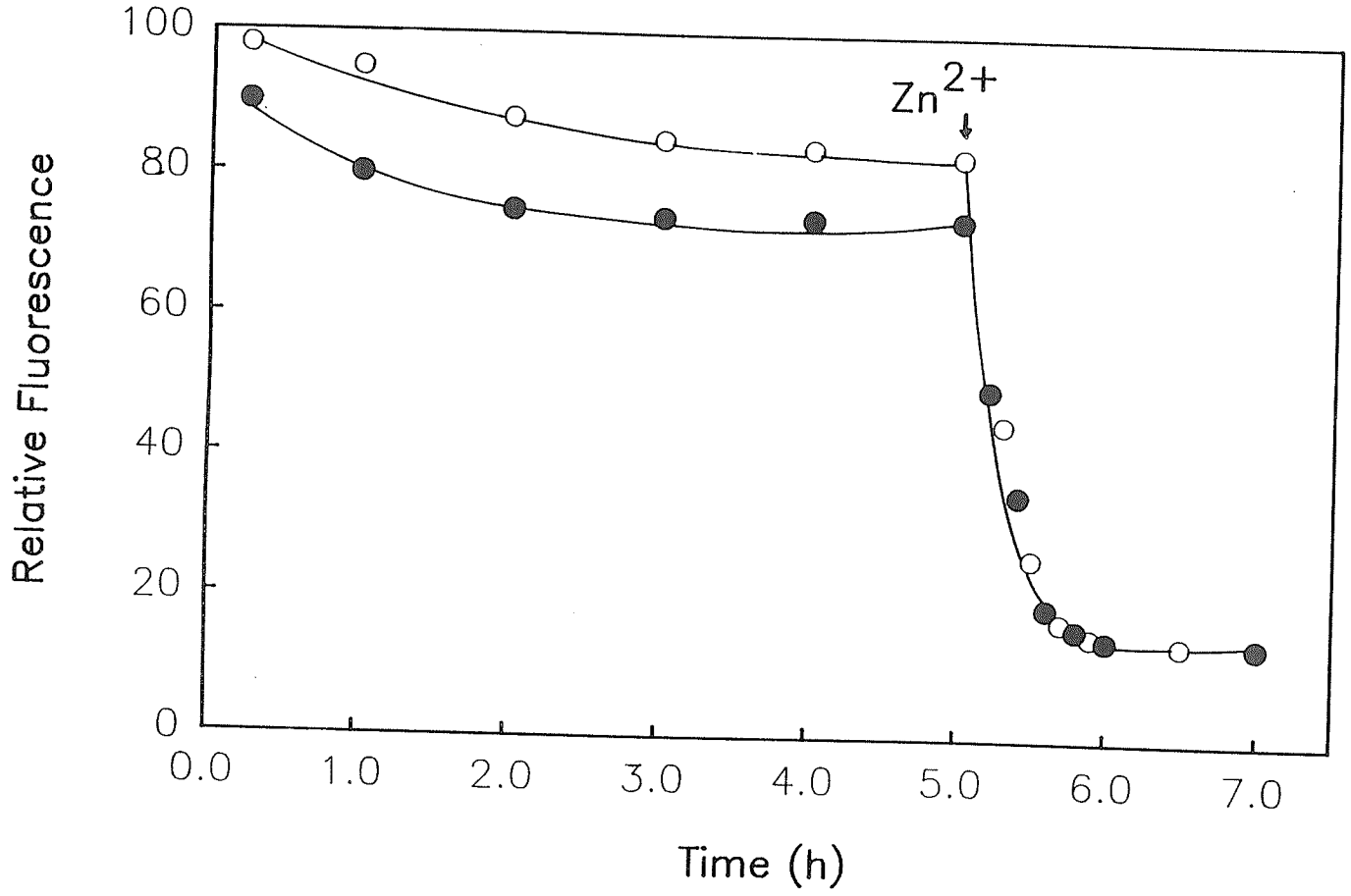

Relative Fluorescence
100
80
60
40
20
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Time (h)
Zn2+

Figure 20. Effect of Zn^{2+} on the reaction of unlabeled and SMNDC-labeled BChE with the carbamoylating agent M7C.

A. Reaction of unlabeled BChE (6.28×10^{-9}M) with M7C (5μM) in the absence (upper trace) and the presence (lower trace) of 1.0×10^{-4}M $ZnSO_4$.

B. As in A, but using SMNDC labeled BChE (4 mol SMNDC/mol BChE)

All reactions were done in 1mM Tris buffer, pH 7.5. Fluorescence λ_{em} 505, λ_{ex} 405.

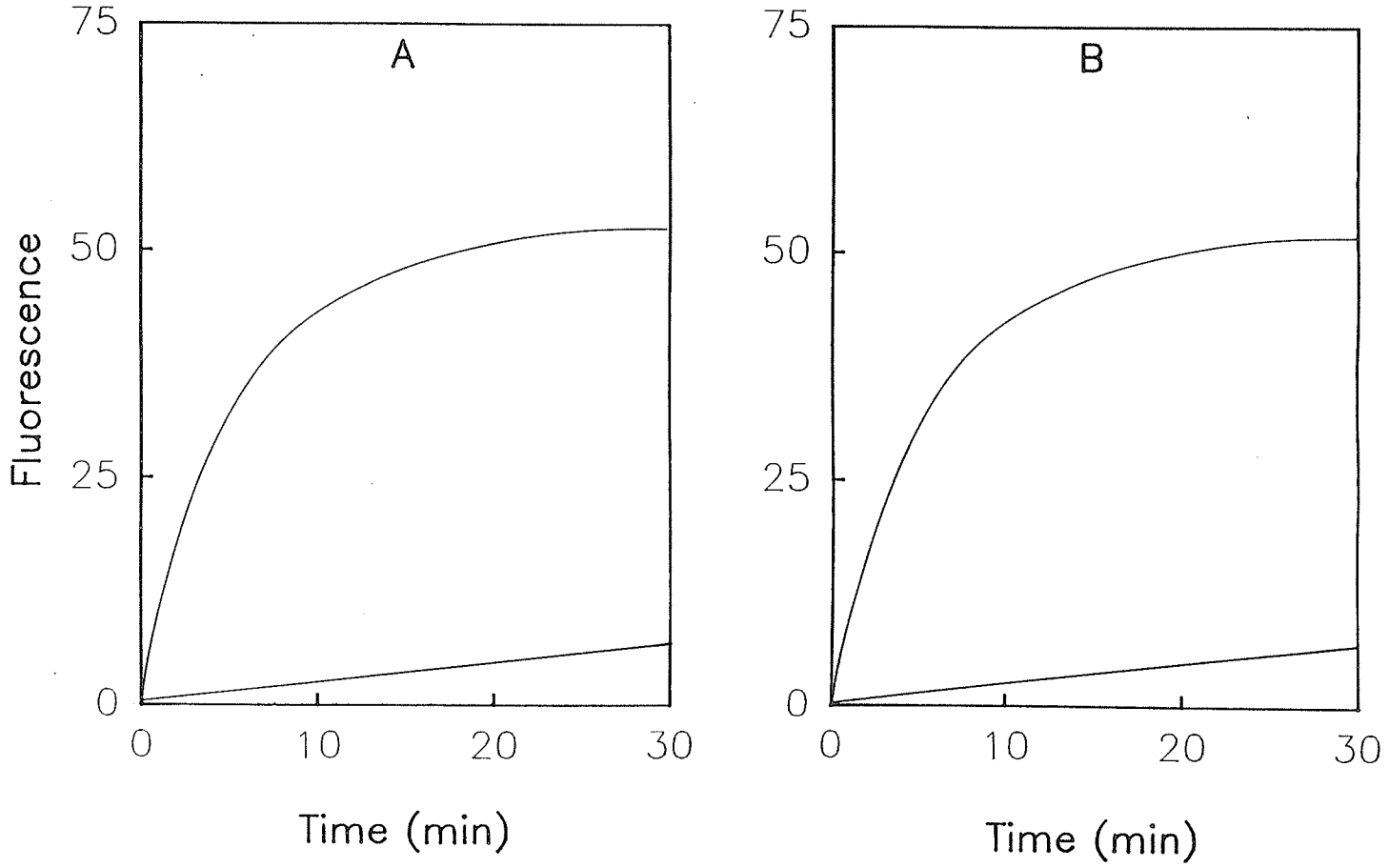
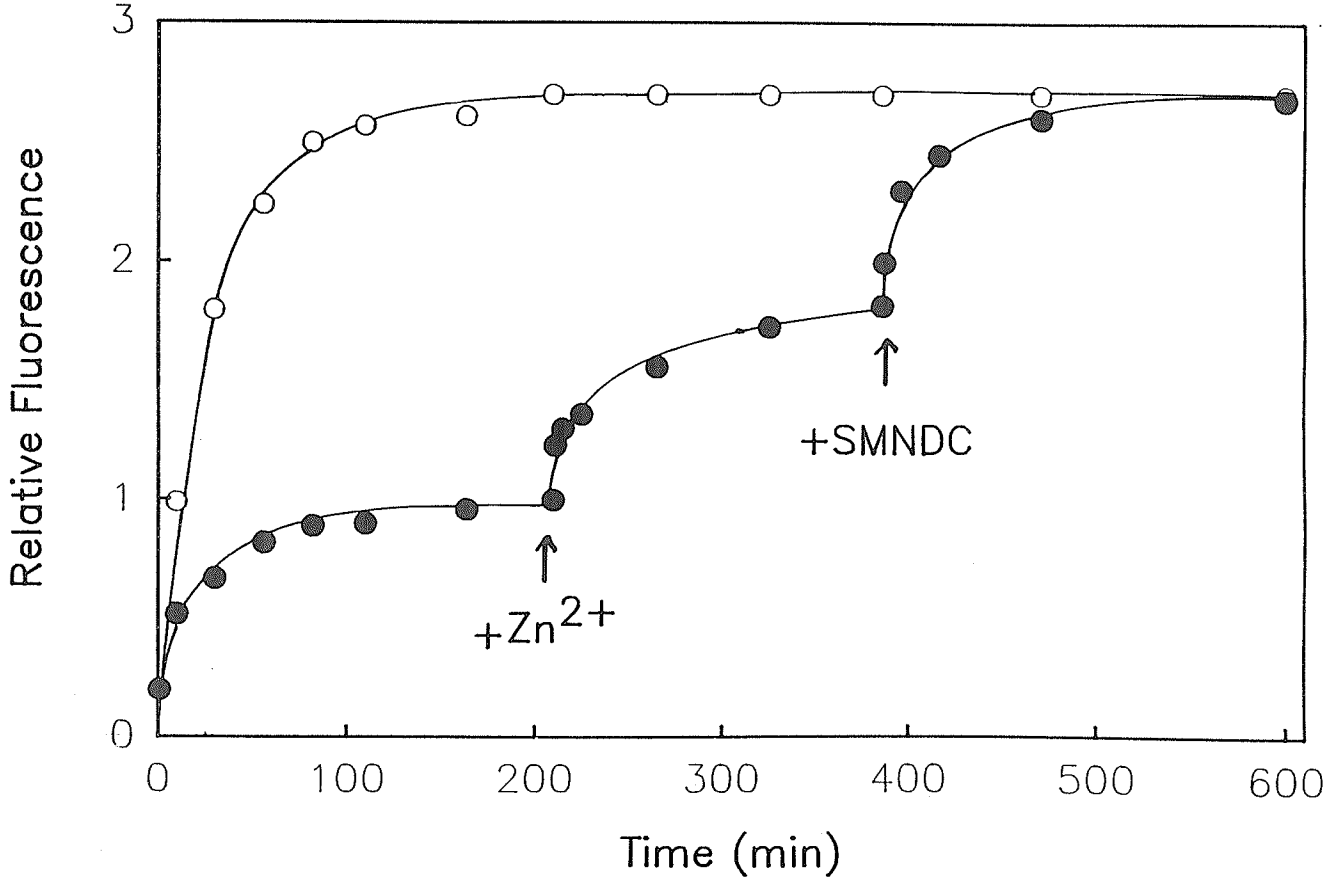

A
B
75
50
25
0
Fluorescence
0
10
20
30
Time (min)

Figure 21. Effects of Zinc on SMNDC fluorescence and reactivity.

(○) 2.45 x 10^{-8}M *T. californica* AChE in 1.0mM Tris/1.0 x 10^{-4}M $ZnSO_4$ preincubated 10 minutes, followed by addition of SMNDC to 1.47 x 10^{-7}M (6:1 label :enzyme). Fluorescence measured at λ_{em}495nm at the intervals indicated.

(●) The same concentration of AChE treated with 9.8 x 10^{-8}M SMNDC (4:1 label:enzyme) and the fluorescence monitored until no further increase. The solution was then made 1.0 x 10^{-4}M in $ZnSO_4$ and the Zn^{2+}-induced increase in fluorescence monitored until constant ,at which point a further 2 mol SMNDC/mol AChE were added and the incorporation of additional label was followed by measuring the fluorescence increase.


Relative Fluorescence
3
2
1
0
0
100
200
300
400
500
600
Time (min)
+Zn2+
+SMNDC

hours. Addition of another 2 mol SMNDC at this point causes a further increase in fluorescence to a level close to that obtained when the enzyme is labeled with 6 mol SMNDC with Zn^{2+} present throughout. All of the bound probe can be removed by reduction with dithiothreitol, indicating that the principal mode of attachment of each SMNDC molecule is through the mercury atom. Other group IIB inactivators (Cd^{2+}, Hg^{2+}) promote similar effects.

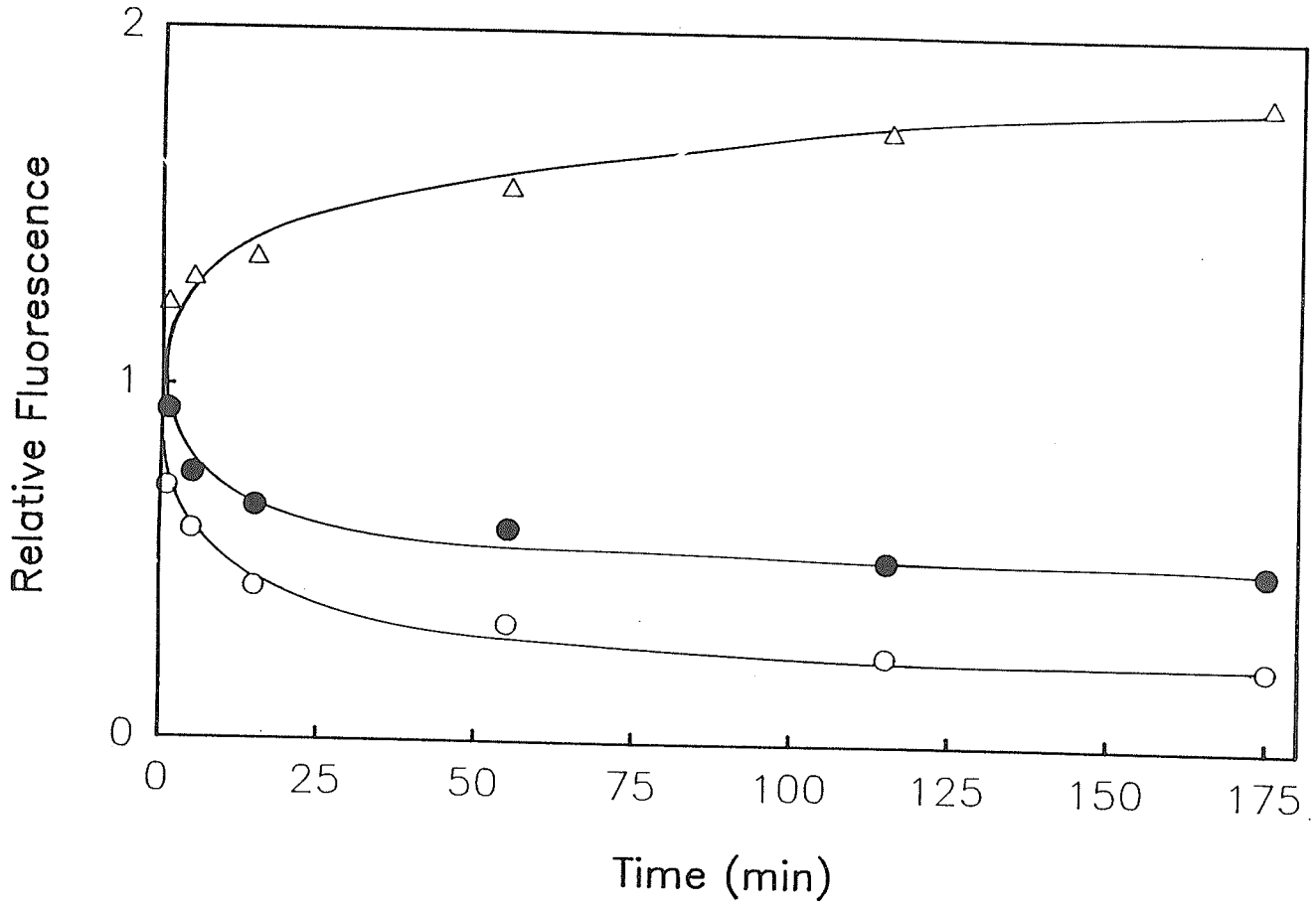
Cations of group IIA and IIB promote opposite effects on the kinetic properties of the enzyme, being activators and inactivators, respectively. Some organic cations such as gallamine and propidium also behave as activators while others (e.g., d-tubocurarine) are inactivators (65,75,76). Fig. 22 shows that, broadly speaking, ligand-induced changes in fluorescence parallel the changes in the kinetic properties of the enzyme. Thus, inactivators such as Zn^{2+} and d-tubocurarine effect an increase in the fluorescence intensity while activators such as Mg^{2+} and gallamine cause a decrease. However, the kinetics of the processes leading to changes in fluorescence are complex and a large portion of the total change in intensity occurs far more slowly than do the previously observed changes in the kinetic properties of the enzyme (65).

Similar experiments were carried out with SMNDC-BChE. While the inactivator Zn^{2+} produced a near-doubling of the fluorescence intensity, as pointed out above, little or no effect on fluorescence was obtained following addition of activators such as Mg^{2+} or gallamine. However, the effects of these species on the kinetic properties of BChE are not known and await further study.

Figure 22. Effects of added cations on SMNDC-AChE fluorescence in 1mM Tris, pH 7.5

Aliquots of a given activator or inactivator at the concentrations indicated below were added to the solution of the complex and the fluorescence monitored until no further changes were observed.

(△) 1.0 x 10^{-4}M $ZnSO_4$
(●) 1.0mM $MgSO_4$
(○) 1.0mM gallamine


Relative Fluorescence
2
1
0
0
25
50
75
100
125
150
175
Time (min)

DISCUSSION

A major goal of the present study was to establish the presence or absence of free thiol groups in cholinesterases from three sources (*T. californica*, *E. electricus*, and human serum). The hypothesis that free thiol groups might be present in these enzymes arose from studies by Mutus (107) on the inactivation of ACHE by Group IIB cations. These species were found to induce a slow conversion of the enzyme to a form best described as "unreactive" towards substrates and acylating agents. Activity could be restored by addition of chelating agents such as EDTA; however, the amount of recoverable activity was found to be inversely dependent upon the time of exposure of the enzyme to these cations prior to the addition of chelators (86, 107). Exclusion of oxygen during the period of exposure to metal ions was found to retard the conversion of the enzyme to the "denatured" (i.e., irreversibly inactivated) state, suggesting that permanent inactivation may be related to the oxidation of a sensitive group on the enzyme, possibly a thiol group.

A search of the extensive acetylcholinesterase literature revealed that over the past five decades, there have been a number of reports that the enzyme is inhibited to varying degrees by a variety of "thiol-specific" reagents, including arsenite (116,117), Lewisite (117-119), maleic acid, iodoacetate and oxidized glutathione (18). Much of the earlier work has been critically reviewed by Mounter and Whittaker (83), Markwardt (120), and Karlin (121), who collectively conclude that

the involvement of essential enzymic thiol groups can be discounted because the concentrations of the inactivating reagents were too high and the rates of inactivation too slow, when compared to "classical" thiol-containing proteins. These conclusions do not apply, however, to proteins which contain free thiol groups which are not directly involved in the biological function of the protein.

More recently, Massoulie and Rieger have reported that *T. californica* AChE is inhibited by *p*-chloromercuribenzoate in a very slow ($t_{1/2}$ approx. 90 min) reaction (22). Wins *et al.* showed that this reagent may interact with AChE at or near a peripheral anionic site as the inhibition can be reversed by addition of the activating cation Ca^{2+}, a peripheral site-specific ligand (122). Wilson and Silman have studied the aresenite inhibition of AChE in detail (84), and concluded that although kinetically consistent with thioarsenate formation, chemical evidence appeared to exclude the formation of this species. Indeed, Wilson and Page have recently shown that arsenite acts by forming a bridged arsenite diester between two adjacent tyrosine residues (123). Finally, it has been reported by Rosenberry (28) that *E. electricus* AChE contains no free sulfhydryl groups on the basis of titrations with radiolabeled N-ethylmaleimide.

In view of these somewhat conflicting data, a more selective thiol reagent bearing a spectroscopic reporter group was sought. N-dansylaziridine is a thiol-selective alkylating agent introduced by Scouten *et al.* (92). This reagent reacted with *T. californica* AChE under conditions of low ionic strength (82). A second class of reactive groups became evident in the presence of inactivators such as Zn^{2+}, and Cd^{2+}. At high ionic strength the reaction of the reagent with

the enzyme was inhibited, suggesting that the enzyme can exist in a variety of ionic strength and/or cation dependent conformational states as hypothesised earlier on the basis of kinetic studies (86). *E. electricus* was reactive towards N-dansylaziridine only in the presence of inactivators. From hydrolysates of labeled enzyme from either source, a fluorescent component having the same thin layer chromatographic mobility as dansyl-S-aminoethylcysteine could be demonstrated. While suggestive of the presence of free thiol groups, these data were not considered to be conclusive, especially in view of the long reaction time required (17 h) and the fact that the reagent has been reported to react with both lysine (124) and methionine (125) residues. A more selective reagent was clearly required.

S-mercuric-N-dansylcysteine (SMNDC) appeared to offer distinct advantages over most of the then-available sulfhydryl reagents. Introduced by Leavis and Lehrer in 1974 (102), SMNDC reacts specifically with thiol groups to form tightly bound mercury-bridged mercaptides. Under suitable conditions, the stoichiometry of the reaction can be determined through exploitation of the spectral changes that accompany the binding of the reagent to the protein. Mutus *et al.* (82) showed that SMNDC reacted under conditions of low and high ionic strength. Again, a further two moles of reagent appeared to be incorporated in the presence of Zn^{2+}. However, this study was open to the criticism that, in view of the highly hydrophobic nature of SMNDC, the interaction of the probe with the enzyme may be due to the tight yet noncovalent binding to a hydrophobic region on the protein surface.

Thus, the present study was initiated in order to resolve these questions with respect to the pssible presence of free sulfhydryl groups in AChE from both *T. californica* and *E. electricus*. The study was extended to include human serum BChE when sequence data indicated the possiblity of a free thiol group in this species also (35). In addition, we wished to examine the potential usefulness of the reagent as a probe of AChE conformation.

Reaction of model proteins with SMNDC

In an attempt to establish criteria for the distinction between noncovalent and covalent interaction of SMNDC with proteins, the reaction of the reagent with a number of test proteins was studied. Proteins were chosen which were readily available in large quantities, were relatively inexpensive and could be obtained in both thiol- and nonthiol containing forms. Thus, both BSA (0.6-0.8 mol SH/mol protein and S-carboxymethyl-BSA (<0.2 mol SH/mol protein) were commercially available. Lysozyme, which has no free sulfhydryl groups, was also commercially available and can easily be reduced according to published methods (109), to give a species which retains catalytic activity but contains two SH groups/mol protein. These four proteins comprise two pairs whose members presumably differ very little in their tertiary structures but are characterized by the absence or presence of free thiol groups. Any differences in their behaviour towards SMNDC can therefore be reasonably supposed to be related to their different thiol content. Their availability in relatively large amounts offered the further advantage that the thiol content estimated

by reaction with SMNDC could be easily verified using the alternative thiol reagent, DTNB, as sulfhydryl group titrant.

The results obtained with BSA and carboxymethyl-BSA do emphasize that caution must be used in interpreting experimental results with SMNDC. Both of these proteins behave in an identical manner towards the reagent, even though only one of them has a free sulfhydryl group. In both cases, bound SMNDC could be removed from the protein by gel chromatography and addition of DTT to either of the probe-protein complexes, which should release free dansylcysteine, had no effect on the fluorescence of the mixture. Thus, in both these cases, the apparently tight interaction of the probe and protein must result from noncovalent forces between the hydrophobic SMNDC molecule and a hydrophobic site on each protein.

In contrast to these observations, the results obtained with lysozyme and reduced lysozyme were more in line with those expected of a pair of analogous nonthiol- and thiol- containing proteins. Thus, no evidence was found for the interaction of SMNDC with lysozyme. Partially reduced lysozyme, on the other hand, produced a slow but characteristic increase in bound dansyl fluorescence, reaching a maximum over a period of 200 minutes. This slow increase in fluorescence appears to be a characteristic of a covalent reaction, since most of the the examples of the use of SMNDC as a thiol reagent found in the literature also were associated with a finite reaction time. (82, 102-106). In addition, the SMNDC-reduced lysozyme complex did not dissociate during gel chromatography whereas reduction of the mercaptide linkage with DTT caused an immediate loss of fluorescence indicative of dissociation of the probe-protein complex.

The above results emphasize the necessity of applying a number of criteria in support of covalent rather than noncovalent interactions of the reagent with proteins. The first of these is a finite reaction time (minutes to hours) as opposed to the instantaneous increase in fluorescence indicative of noncovalent binding. This fact alone is insufficient, since the slow reaction time probably reflects a relatively inaccessible location of the thiol group; surface-located thiols should react rapidly. The second involves reduction of the mercaptide linkage with an excess of dithiothreitol, which should result in a decrease in fluorescence to control (absence of protein) levels. Since there remains the possibility that the dansylcysteine moiety might still be bound to the protein even after reduction, a third criterion should be applied, namely, failure to separate bound probe from protein by chromatography or electrophoresis.

Reaction of AChE and BChE with SMNDC

Application of the above criteria to the reaction of SMNDC with affinity-purified AChE from *T. californica* confirmed the earlier finding of Mutus *et al.* (82) that this enzyme contained one free sulfhydryl group per subunit. During the course of the study, this result was further verified following publication of the amino acid sequence of the enzyme (39). The free sulfhydryl is located at Cys-231 of the 575 amino acid-containing polypeptide. Similarly, the present study has demonstrated that human serum cholinesterase reacts stoichiometrically and covalently with SMNDC, indicating that the putative sulfhydryl group at Cys-66 (35) is present in the free thiol form. Under

conditions where both *T. californica* AChE and human serum BChE incorporate 4 mol SMNDC/mol enzyme, *E. electricus* AChE does not react with SMNDC. In this case, the finding of no SMNDC-reactivity is consistent with there being no free sulfhydryl groups present, as previously reported (28).

It is interesting that both *T. californica* AChE and BChE do not appear to react with most of the more conventional thiol reagents. Cys-231 of the *Torpedo* enzyme reacts with monobromobimane, another fluorescent sulfhydryl-specific reaget, only under strongly denaturing conditions (39), while Cys-66 of BChE does not react under any conditions with iodoacetic acid (35). In our laboratory, the only other reagent found capable of reaction with these enzymes was DTNB. However, low protein concentrations (solubility considerations limit the maximum protein concentrations to $<1\mu M$) render these results at best tentative. It may be concluded that the free SH group in *T. californica* AChE and in human serum BChE is located in an environment that permits access only to those probes which contain a hydrophobic group such as the dansyl moiety of SMNDC. Although the high degree of sequences homology and similar placement of the intrasubunit disulfide bonds are indicative of similar folding patterns, the quite dissimilar location of the free SH group in these enzymes likely results in a somewhat different environment in this region of the enzyme surface to which SMNDC binds. These differences give rise to the different fluorescence quantum yields, with the result that SMNDC-BChE has only about half of the fluorescence intensity shown by SMNDC-AChE.

The nature of the additional groups in the *Torpedo* enzyme, which

react with SMNDC in the presence of Zn^{2+} remains obscure, although sequencing studies rule out the presence of additional thiol groups on the catalytic subunit. MacPhee-Quigley *et al.* (39) also reported the presence of an additional, unidentified component labeled by monobromobimane. The formation of Hg(II) complexes is favoured with but not restricted to sulfur-containing ligands and it is possible that appropriately-positioned O- or N-containing groups become accessible in the presence of Zn^{2+}. Alternatively, the additional SMNDC-reactive groups may in fact be located on the "structural subunit" reportedly present in variable amounts in this enzyme.(126). However, this does not seem likely since *E. electricus* AChE, which does not have structural subunits and does not react with SMNDC in low or high ionic strength media, does exhibit a Zn^{2+}-induced reaction with N-dansylaziridine. Further studies will be necessary in order to clarify this point.

The free SH group of either *T. californica* AChE or human serum butyrylcholinesterase is clearly not involved in the catalytic function of the enzyme, since the SMNDC-labeled enzymes retain almost full activity. This residue does not appear to have been conserved since the free sulfhydryl group of butyrylcholinesterase is at position 66 in the primary sequence , at position 231 in *T. californica* AChE, and is absent in *E. electricus* AChE. The location of the free sulfhydryl group, if any, in *Drosophila melanogaster* acetylcholinesterase (41) is not known but there is no cysteine residue in the vicinity of either residue 66 or 231 which might be a possible candidate. Cys-231 is also ruled out as the site of binding of Zn^{2+} ions, a process which results in a slow, reversible inactivation of the enzyme (66,67). SMNDC-

labelled enzyme is as sensitive as the unmodified enzyme towards inactivation by Zn^{2+}, and the kinetics of inactivation in the presence and absence of SMNDC are identical.

SMNDC has potential utility in the investigation of ligand-induced conformational changes in acetylcholinesterase. The fluorescence intensity obtained when the enzyme is stoichiometrically labeled differs according to the ionic composition of the medium used, decreasing in the order 1.0 mM Tris/10^{-9} M Zn^{2+}> 1.0mM Tris> 1.0mM Tris /0.1M Na^{+}. These differences are thus in accord with the previously reported effects of these species on the catalytic properties of the enzyme and are broadly consistent with the postulated "unreactive", "active" and "activated" states, respectively, of the enzyme (66,67). Similarily, addition of Zn^{2+} to SMNDC-labeled enzyme which had been prepared in the absence of this cation produced an increase in SMNDC fluorescence, whereas Mg^{2+} and gallamine do promote an initially rapid change in fluorescence, most of the increase or decrease takes place over a period of several hours. It thus appears likely that SMNDC is monitoring conformational changes that are in addition to those which result in alterations in the catalytic properties of the enzyme and which occur within a longer time scale relative to both the inactivation and activation processes. A more detailed analysis of the kinetics of the ligand-induced changes in the fluorescence of SMNDC-AChE will be required in order to determine if this probe is a useful reporter of conformational changes relevant to alterations in catalytic function.

SMNDC-labeled human serum cholinesterase exhibits a spectrum of behaviour towards added ligands which is both similar to and different

from that found with SMNDC-AChE. Exposure of the complex to Zn^{2+} results in an enhancement of fluorescence only, without the incorporation of additional label that is found with AChE. Mg^{2+}, and gallamine, which promote a decrease in SMNDC fluorecence, actually promote an increase in SMNDC-BChE fluorescence. Furthermore, the kinetics of these processes are quite different, with the Mg^{2+}-stimulated increase being initially rapid, followed by a slower second phase of fluorescence increase. With gallamine, the order of these processes is reversed, there being an inital slow phase followed by a progressivley more rapid increase in fluorescence. It would be premature to speculate on the origins of these differences, particulary since the effects of these ligands on the catalytic properties of BChE have not yet been studied.

In conclusion, this study has demonstrated that, provided appropriate precautions are observed, SMNDC can be usefully employed as a probe for the detection of protein-bound sulfhydryl groups. In the case of *T.californica* AChE and human serum BChE, the reagent successfully labeled sulfhydryl groups where more conventional thiol-selective reagents failed. The finding of free sulfydryl groups in both of these enzymes was verified elsewhere by sequencing studies. The reagent showed some promise as a probe of ligand-induced conformational changes in AChE; however, further study is required in order to establish any correlation between the observed fluorescence changes and changes in the kinetic properties of the enzyme.

PART II

STUDIES ON ARSENIC ANALOGUES OF CHOLINE

AND

ACETYLCHOLINE

INTRODUCTION

It has been suggested that in a "normal" biochemical cycle, the cholinergic agonist acetylcholine is rapidly metabolized upon its release from the cholinergic receptor. Incomplete metabolism of this agonist is thought to cause or contribute to a variety of neuropsychiatric disorders such as Huntington's Chorea, Alzheimers dementia, and childhood hypotonic schizophrenia (130).

Potter, in 1933, first recognized hypotonic schizophrenia in children. His observations of children living in institutions led him to conclude that childhood schizophrenia was not as rare a disorder as was originally thought (131). It was Bender however, in 1947, who was the first to suggest that childhood hypotonic schizophrenia was associated with a dysmaturation process of the cholinergic system. He based this conclusion on observations of certain similarities between the schizophrenic child and a newborn baby. A newborn baby has impaired ability to metabolize acetylcholine, which manifests itself in the following ways: soft muscle tone, lax joints, pale and soft velvety skin, and lack of ability to control body temperature. All of these symptoms are found in schizophrenic children. Early investigators of schizophreniza also noted that some patients developed hyperthermia in overheated rooms. Since hyperthermia could be induced by atropine, (a known cholinergic antagonist), the involvement of the cholinergic system in schizophrenia was again implicated.(132)

Neubauer, in 1975, suggested that hypotonic schizophrenia was related to a failure of cholinergic transmission in the cortex. He observed that Ditran, a powerful anticholinergic, caused psychomimetic effects which were reversed in the presence of tetrahydroaminocrine, a powerful anticholinesterase (133). Subsequent studies by Rosenthal and Bigelow (134) also supported these findings, in that they observed neostigmine and physoistigmine had the ability to increase cholinergic activity by virtue of the inhibition of acetylcholinesterase. A recent comparison of the syptoms of childhood hypotonic schizophrenia, with those symptoms of the "cholinergic syndrome" led Cantor *et al.* to suggest that the cholinergic system be investigated in order to clarify its role, if any, in hypotonic schizophrenia (135).

The cholinergic system is essential to life, and does not lend itself easily to manipulation. Unlike other neurotransmitters such as dopamine, norepinephrine and serotonin which either are, or can be made to become, fluorescent, acetylcholine possesses no convenient properties which would permit examination of its distribution *in vivo*. It is known that acetylcholine has the ability to influence the activity of a variety of target tissues. These influences are usually expressed in a variety of ways, including secretions, muscular contractions and bradycardia. Acetylcholinesterase is the only known cholinolytic agent, and almost all the acetylcholine released from nerve endings is rapidly hydrolyzed soon after dissociation from acetylcholine receptors (136).

The pharmacological effects of acetylcholine are traditionally classified as being either nicotinic or muscarinic, a system based on two broad classes of ACh receptor defined by specific interaction with

the potent alkaloid agonists nicotine and muscarine, respectively. Differences in the interactions of acetylcholine with respect to nicotinic or muscarinic acetylcholine receptors are postulated to arise from the interaction of the receptor with different combinations of the functional groups in the acetylcholine molecule. The nicotinic receptor is believed to interact with both the quaternary ammonium and carbonyl groups, whereas the muscarinic receptors interact with the ester oxygen and the methyl group (136).

Since it is the muscarinic acetylcholine receptor which plays an essential role in many physiological and behavioural responses, it has been suggested that a decrease in these receptors in the brain plays a significant role in elderly patients with Huntington's Chorea, Alzheimer's dementia, and hypotonic schizophrenia. Until recently, however, the only certain way to determine the involvement of the muscarinic acetylcholine receptor in the aforementioned disorders, was through autopsy studies in humans or animals (137). There is thus a clear need for a means of assessing cholinergic involvement *in vivo*.

To date, the most successful attempts to label these receptors has involved the use of radiotracers with external imaging processes such as positron emission tomography (PET) and the related single photon emission computed tomography (SPECT). The paucity of data in this area is a direct reflection of the severe difficulties involved in the synthesis of appropriate bioactive molecules containing a suitable radionuclide (usually ^{99}Tc or ^{123}I). However, muscarinic cholinergic receptors have been successfully imaged using ^{123}I-3-quinuclidinyl-4-iodobenzilate (IQNB), a muscarinic antagonist (137,138). A note of caution was sounded by Schwartz (139), who showed by autoradiographic

methods that the distribution of ^{3}H-ACh in the brain was significantly different from that of muscarinic agonists such as ^{3}H-QNB and ^{3}H-N-methylscopolamine or nicotinic antagonists such as ^{125}I-α-bungarotoxin.

A potential alternative to these high affinity radiotracers might involve the synthesis of acetylcholine analogues as false cholinergic neurotransmitters to image the cholinergic system. Comparative studies of acetylcholine and certain of its analogues, were conducted in the 1930's. Equivalent concentrations of the nitrogen, phosphorus and aresenic analogues were exposed to the action of equal amounts of hemolyzed human blood (i.e, to what is essentially the action of serum cholinesterase). It was noted that the enzyme was able to function as effectively with the P and the As analogues as with acetylcholine, although detailed quantitative studies were not carried out (140). Pharmacological data on these compounds are also sparse, with only one early study reporting that acetylarsenocholine elicited about 1% of the acetylcholine response on various tissue preparations (141).

Arsenocholine has been known to exist naturally in various edible shellfish since 1919. It is only recently, however, that attempts have been made to determine the effects of arsenocholine on humans after ingestion of the shellfish. It had been observed that after a shellfish dinner, there were elevated levels of arsenic in the urine of people whose diet contained shellfish as a major component. Comparative studies by Coulson (142), using humans and rats, clearly indicated that the arsenic compounds were cleared by the kidneys at a rate of 70% in 24 hours. It was concluded that the arsenic compounds are present in a non-toxic form, and could be rapidly excreted. It wasn't until 1977 that Cannon *et al.* (143) elucidated the structure of

the non-toxic form as arsenobetaine, in both rock lobster and human urine. Since then, a variety of shrimp, dusky shark, sand whiting and fishmeal have also been shown to contain arsenobetaine. In addition, arsenosugars, arsenocholine and acetylarsenocholine have been identified as naturally occuring organoarsenicals.

The observation that organoarsenicals occur naturally has renewed interest in the biological effects of these compounds. Studies by Hedlund *et al.* (144), using rat brain and diaphragm preparations to assess muscarinic and nicotinic function, *T. marmorata* homogenates for cholinesterase activity, bovine brain for choline acetyltransferase activity, and guinea pig ileum conraction as an estimate of postsynaptic muscarinic agonist activity, indicated that indeed acetylarsencholine behaved as a cholinergic ligand in all of these areas, but with widely differring efficacies with respect to acetylcholine. Thus, while acetylarsenocholine was equipotent with acetylcholine as a nicotinic agonist in rat medulla-pons and as a muscarinic agonist in rat cerbral cortex, the compound elicited only about 1% of the acetylcholine response in guinea pig ileum (muscarinic) and rat diaphragm (nicotinic) preparations. Arsenocholine was shown to be a substrate for choline acetyltransferase (K_m 240 mM; K_m for ACh about 20 mM) and acetylarsenocholine was found to be hydrolyzed by AChE (again, no quantitative data reported).

In view of the lack of data on the pharmacological effects of the arsenic analogues of arsenocholine and acetylarsenocholine, and given the tantalizing possibility that these compounds might bind tightly to at least some acetylcholine receptors without eliciting an appreciable pharmacological response, it was felt that these compounds merited

further investigation. The report by Goetz and Norin on the chemical synthesis of these compounds (145) makes feasible the preparation of analogs containing ^{71}As, a positron emitter with potential utility in the imaging of cholinergic systems *in vivo*. Thus, as part of a broader collaborative project having these goals in mind (with M. Billinghurst and D. Abrams, Dept. of Radiopharmacy, Faculty of Medicine, University of Manitoba), we undertook to assess the properties of arsenocholine as an inhibitor of AChE and to determine the kinetics of hydrolysis of acetylarsenocholine as a pseudosubstrate of the enzyme. The structures of these compounds are shown in Fig. 23.

Figure 23. Structure of a): arsenocholine and b): acetylarsenocholine.

$(CH_3)_3AsCH_2CH_2OH$ a

$(CH_3)_3AsCH_2CH_2OCOCH_3$ b

MATERIALS

Materials	Supplier
Crude 11S *E.electricus* AChE Type VI-S	Sigma Chemical Company St. Louis, Missouri, U.S.A.
Acetylthiocholine chloride	
DTNB	
Arsenocholine and acetylarsenocholine	Were a generous gift from Dr. D. N. Abrams, Dept. of Radiopharmacy, University of Manitoba.

Ellman assay buffer: 0.10M NaH_2PO_4, pH 8.0.

5,5'-dithiobis-(2-nitrobenzoic acid): 0.01M DTNB in Ellman buffer

acetylthiocholine (ASCh): 3.2mM in water. Prepared fresh for each series of experiments.

0.05M solutions of arsenocholine and acetylarsenocholine were prepared in water.

All buffers used in these studies were prepared from glass-distilled deionized water.

METHODS

Purification of *E. electricus* AChE

Crude *E. electricus* AChE was purified by p-PTA affinity chromatography as described in Part I, and diluted to 55 U/mL for use in the assay.

Ellman assay

The Ellman assay was performed as described in Part I. A modified assay was performed using 3.2mM acetylthiocholine as a substrate, over a range of 10-200μM. All assays were carried out in triplicate using a Hewlett-Packard HP8452A diode array spectrophotometer. All the assays were carried out in the absence and presence of two concentrations of inhibitor (arsenocholine or acetylarsenocholine). The enzyme stability was monitored by Ellman assay every half hour, and appropriate corrections were applied to all measured rates.

Data analysis for the Ellman assay.

The rates obtained in the presence and absence of inhibitor were subjected to a nonlinear least squares data analysis using the computer programme SSKINFIT (G. Tomlinson, unpublished). This programme allows rapid analysis of kinetic data in terms of six classical inhibition

models (Competitive, Uncompetitive, Noncompetitive, Mixed, Hyperbolic Competitive, and Hyperbolic Noncompetitive). Goodness of fit is assessed by the comparison of the sums-of-squares of residuals and by examination of Lineweaver-Burke plots.

pH stat assay

The pH stat assay was conducted under anaerobic conditions, using a Radiometer Type 11 titrimeter. Anaerobic water (0.1M NaCl), 5μL enzyme (55u/mL), and inhibitor were mixed, and allowed to incubate and reach a pH level of 8.0 at room temperature for 30s. To this mixture was added substrate over a concentration range of 10-100μM. The rate was measured by NaOH titration of the protons released during the reaction. Data were analyzed graphically by means of Lineweaver-Burke plots. The quality of the data was insufficient to allow detailed analysis of the effects of arsenocholine as an inhibitor of either acetylcholine or acetylarsenocholine hydrolysis.

RESULTS AND DISCUSSION

The muscarinic cholinergic ligands, acetylarsenocholine (AcAsCh) and arsenocholine (AsCh), are potential inhibitors of *E.electricus* AChE. The kinetics of the hydrolysis of acetylthiocholine (ASCh) in the absence and presence of AsCh and AcAsCh, plotted in Lineweaver-Burk form, are shown in Figs. 24 and 25, respectively. Both compounds were found to be moderately potent competitive inhibitors of the hydrolysis of this substrate by AChE. Nonlinear least squares analysis of the data obtained in the absence of inhibitor gave a value for K_m of 130 μM, in reasonable agreement with previously reported data (28). Using this value and the value of V_{max} obtained in the same experiment as constants, the data obtained in the presence of either inhibitor were simultaneously fitted to the competitive inhibition model and best fit values of the competitive inhibition constants (K_i's) were estimated. For AsCh, K_i was 96.2 μM while AcAsCh gave a K_i of 42.3 μM. The lower value obtained with the latter compound likely reflects the fact that AcAsCh is also a substrate and interacts more strongly with the enzyme than its hydrolysis product AsCh.

The hydrolysis of acetylcholine (ACh) was compared to the hydrolysis of acetylarsenocholine by AChE, using a pH-stat assay (Figure 26). Nonlinear least squares analysis shows that these compounds are almost equipotent as substrates, ACh having a K_m of 408 μM while AcAsCh had a K_m of 482 μM. Similarly, the ratio of V_{max} values (ACh/AcAsCh) was about 1.14. These results provide quantitative

Figure 24. Lineweaver-Burk plots of the effects of the inhibitor arsenocholine using 3.2mM acetylthiocholine as substrate in a modified Ellman assay.

(●) no inhibitor
(□) 1 x 10^{-4}M arsenocholine
(▼) 2 x 10^{-4}M arsenocholine
(■) 4 x 10^{-4}M arsenocholine
(△) 8 x 10^{-4}M arsenocholine

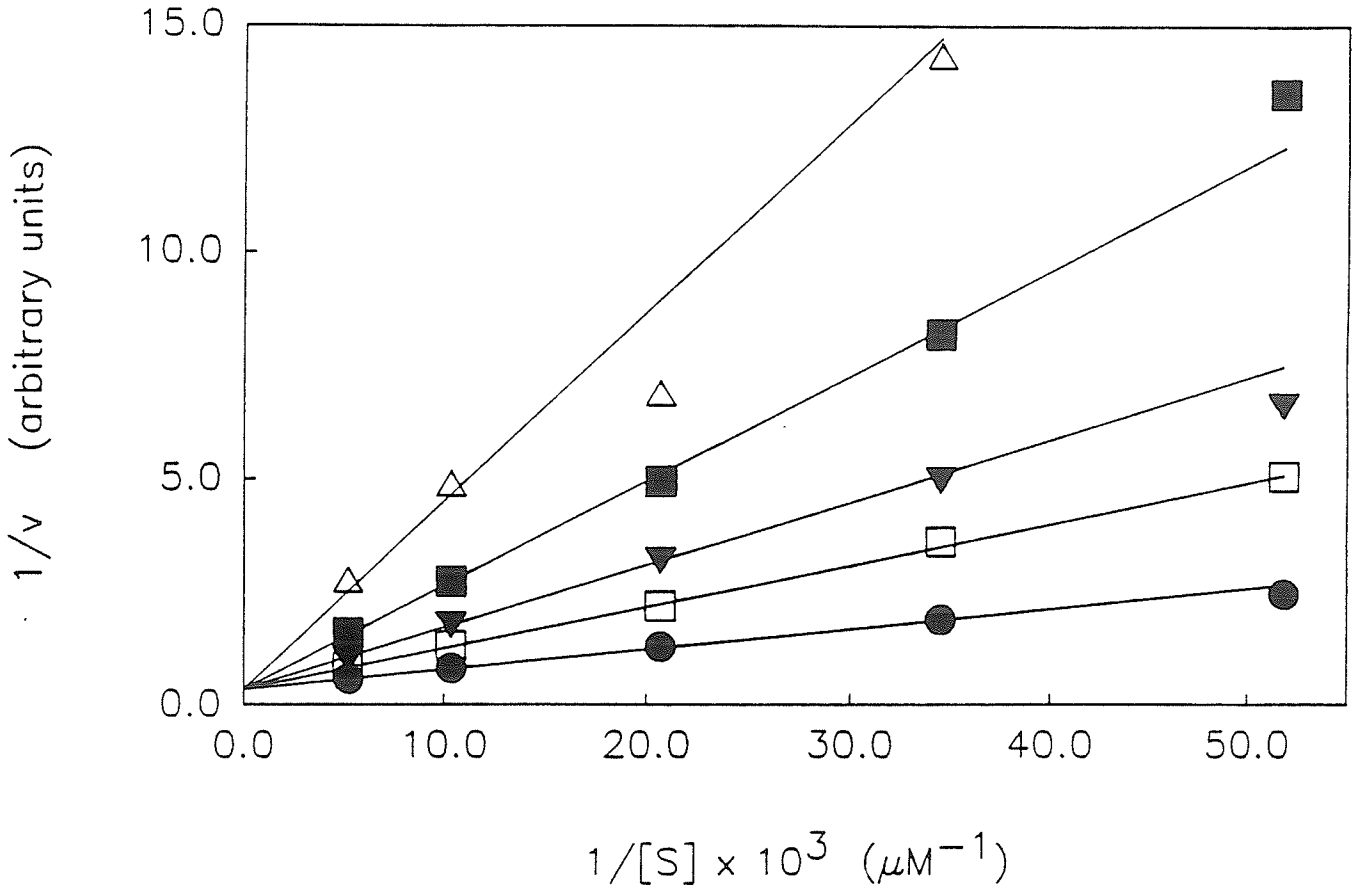
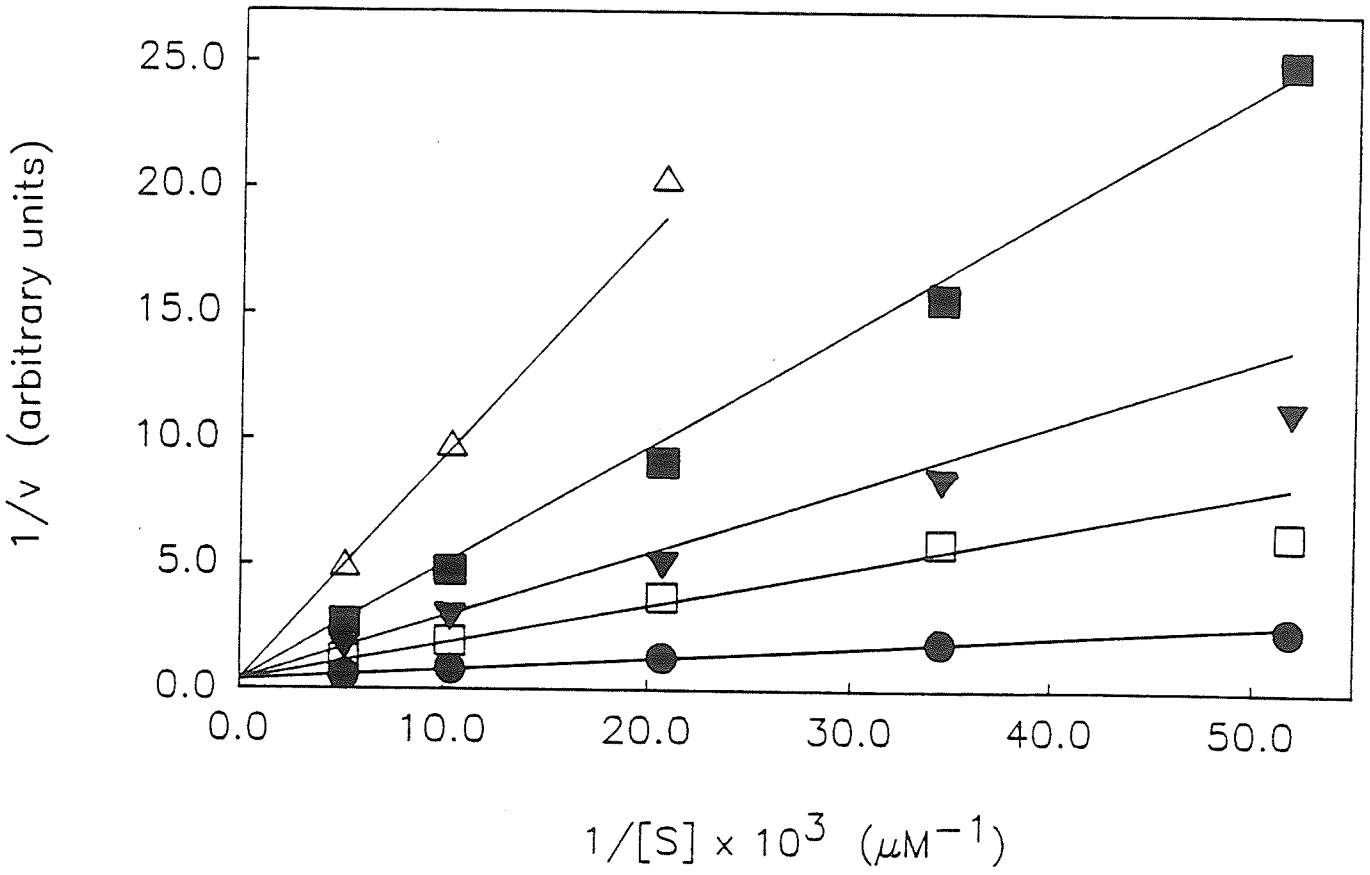

15.0
10.0
5.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
1/v (arbitrary units)
1/[S] x 10^3 (μM^-1)

Figure 25. Lineweaver-Burk plots of the effects of the inhibitor acetylarsenocholine using 3.2mM acetylthiocholine as substrate in a modified Ellman assay.

(●) no inhibitor
(□) 1 x 10^{-4}M acetylarsenocholine
(▼) 2 x 10^{-4}M acetylarsenocholine
(■) 4 x 10^{-4}M acetylarsenocholine
(△) 8 x 10^{-4}M acetylarsenocholine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20.0
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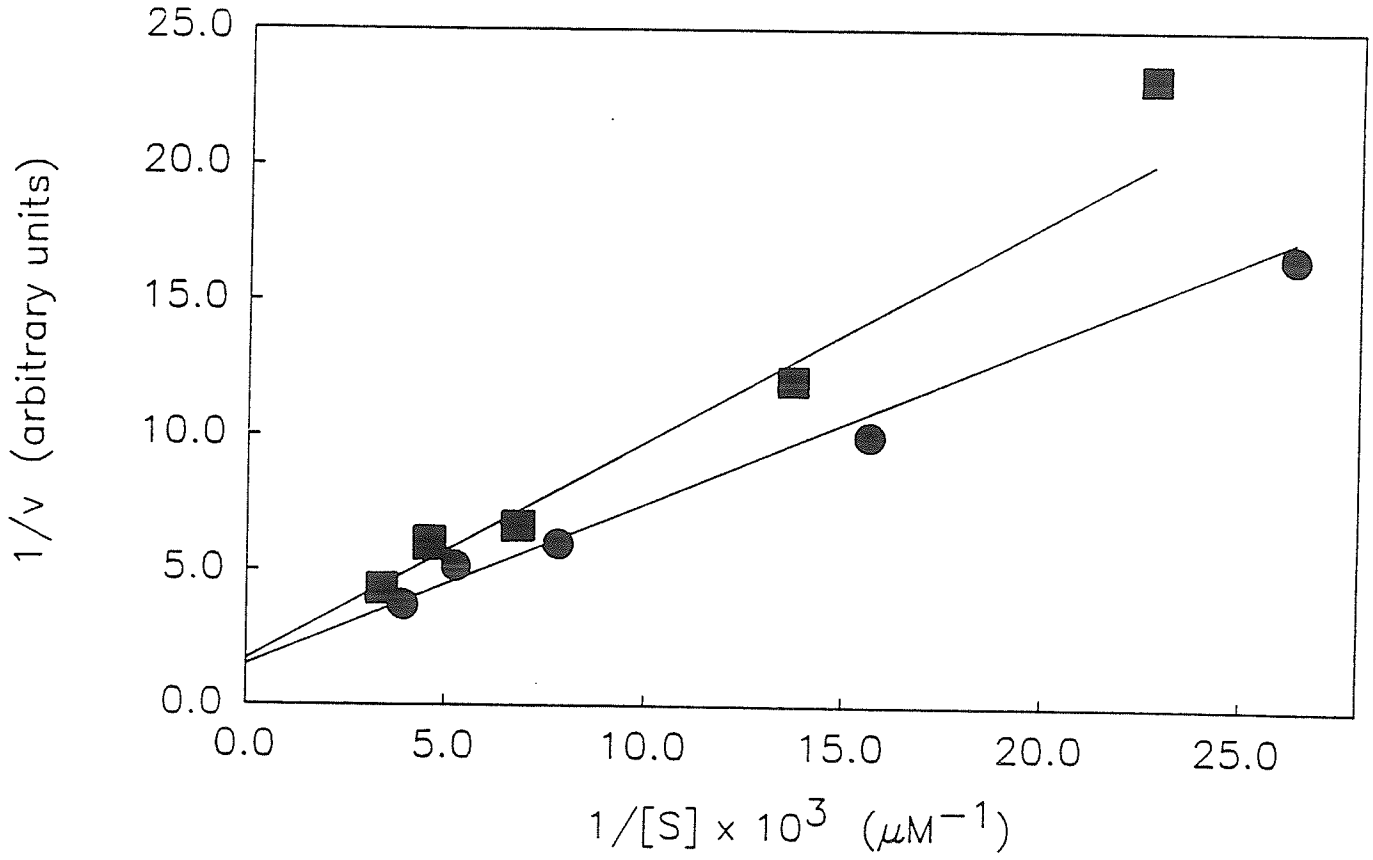
10.0
5.0
0.0
1/v (arbitrary units)
0.0
10.0
20.0
30.0
40.0
50.0
1/[S] x 10^3 (μM^-1)

Figure 26. Determination of Km for both acetylcholine (●) and acetylarsenocholine (■) using *E.electricus* AChE.

The Km of acetylcholine was determined to be 3.03×10^{-4}M by extrapolation, and the Km for acetylarsenocholine was determined to be 3.22×10^{-4}M by extrapolation.


25.0
20.0
15.0
10.0
5.0
0.0
1/v (arbitrary units)
0.0
5.0
10.0
15.0
20.0
25.0
1/[S] x 10^3 (μM^-1)

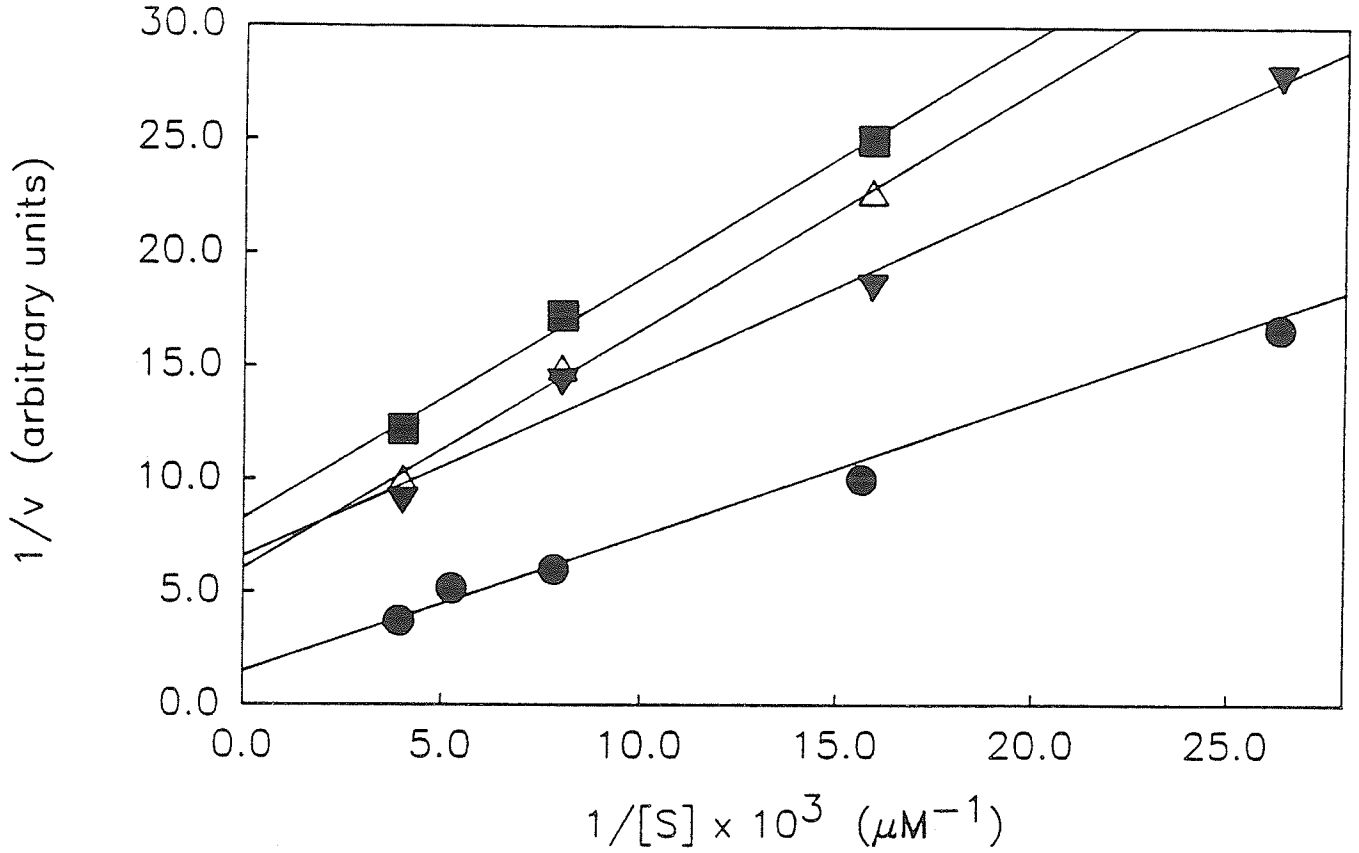
confirmation of the early qualitative findings of Roepke and Welch (143) that acetylarsenocholine is a cholinergic ligand which has a rate of hydrolysis similar to that of acetylcholine (although the enzyme used was serum cholinesterase).

Attempts were also made to examine the effects of AsCh and choline as inhibitors of ACh and AcAsCh hydrolysis, respectively. Technical difficulties with the pH-stat, the availability of insufficient amounts of the arsenic analogs and time constraints conspired to prevent the acquisition of data of sufficient precision for meaningful analysis of the results. In one such study, the inhibition of acetylcholine hydrolysis by arsenocholine appeared to possess a sizeable uncompetitive component (Fig. 27). Nonlinear least squares analysis indicated that these data were best described by a hyperbolic (partial) noncompetitive model, as assessed by the sum of squares of residuals. However, the fit may be seen to be not very good and the significance of the uncompetitive component of inhibition is unclear. The kinetic parameters obtained in this portion of the study are summarized in Table II.

Given that acetylcholine and acetylarsenocholine are almost equipotent as substrates of AChE, it is a little surprising that the pharmacological properties of the two compounds differ so markedly. As mentioned earlier, AcAsCh has only about 1% of the activity of ACh as expressed in a variety of pharmacological tests of cholinergic function (144). The differences between acetylarsenocholine and acetylcholine, perhaps may be attributed to conformation differences between the two compounds. Preliminary crystal data (T. Secco, unpublished data), for AcAsCh, have shown that acetylarsenocholine, like acetylcholine (137,

Figure 27. Inhibition of acetylcholine hydrolysis by arsenocholine using the pH-stat assay.

(●) no inhibitor
(■) 1.99×10^{-3}M arsenocholine
(△) 0.995×10^{-3}M arsenocholine
(▼) 0.0498×10^{-3} arsenocholine


30.0
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0.0
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5.0
10.0
15.0
20.0
25.0
1/v (arbitrary units)
1/[S] x 10^3 (μM^-1)

TABLE II. Summary of kinetic constants obtained with ASCh, ACh, AsCh and AcAsCh.

Substrate	Inhibitor	K_m (μM)	K_i (μM)	Best fit model
acetylthiocholine	none	130.0+/-8.4	----	Michaelis-Menten
"	arsenocholine	----	96.2+/-1.8	Competitive
"	Ac-arsenocholine	----	42.3+/-0.9	Competitive
acetylcholine	none	407.8+/-121.1	----	Michaelis-Menten
Ac-arsenocholine	none	481.9+/-268.8	----	Michaelis-Menten
acetylcholine	arsenocholine	----	144.6+/-56.9	Hyperbolic Noncompetitive (β=0.30)

145), has a gauche conformation about the As-CH_2-CH_2-O group. However, the torsion angle for acetylarsenocholine is +58 degrees, whereas it is +77 degrees for acetylcholine. The CH_3-As-CH_3 angle in AcAsCh is smaller than the CH_3-N-CH_3 angle in ACh. The CH_3-N- CH_2-, N- CH_2-CH_2, CH_2-CH_2-O and O-C=O angles are all larger for acetylcholine than the corresponding angles in acetylarsenocholine. These minor differences could contribute to the differences in the way in which acetylcholine and acetylarsenocholine interact with acetylcholine receptors. Because the differences are so minor, the two compounds would be expected to show the observed similarity in binding properties. It is, however, the subsequent coupling steps involved in the cholinergic response which could be affected (143).

In summary, arsenocholine and acetylarsenocholine are both moderately potent competitive inhibitors of acetylthiocholine hydrolysis. In addition, acetylarsenocholine is hydrolyzed by AChE with an efficiency comparable to the natural substrate acetylcholine. The low cholinergic pharmacological potency of these compounds combined with their near equipotent binding to acetylcholine receptors hold the promise that arsenocholine might be worthy of investigation (in suitable isotopic form) in the imaging of these receptors *in vivo*.

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PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK

ARTICLES IN REFEREED JOURNALS

1. G. Tomlinson & E. M. Kinsch. S-mercuric-N-dansylcysteine labels the free sulphydryl groups of human serum cholinesterase. Biochem. Biophys. Res. Commun. 158, 503-507 (1989).

2. G. Tomlinson & E. M. Kinsch. The reaction of S-mercuric-N-dansylcysteine with acetylcholinesterase and butyrylcholinesterase. Biochem. Cell Biol. 67, 377-344 (1989).

ABSTRACTS AND PRESENTATIONS

1. E. M. Kinsch & G. Tomlinson. The reaction of acetylcholinesterase with thiol-selective reagents. Proc. Can. Fed. Biol. Soc. 29, 197 (1986).

2. G. Tomlinson & E. Kinsch. Probes of acetylcholinesterase function. Abstr. 14th Int. Congr. Biochem. Th-317 (1988).