Title
Native forms of electric eel acetylcholinesterase

Author
Parker, Keith Krom

Publication Date
1978

Peer reviewed|Thesis/dissertation

NATIVE FORMS OF ELECTRIC EEL ACETYLCHOLINESTERASE: PURIFICATION AND ACTIVE SITE STUDIES

by

Keith Krom Parker
B.S., Montana State University 1972

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

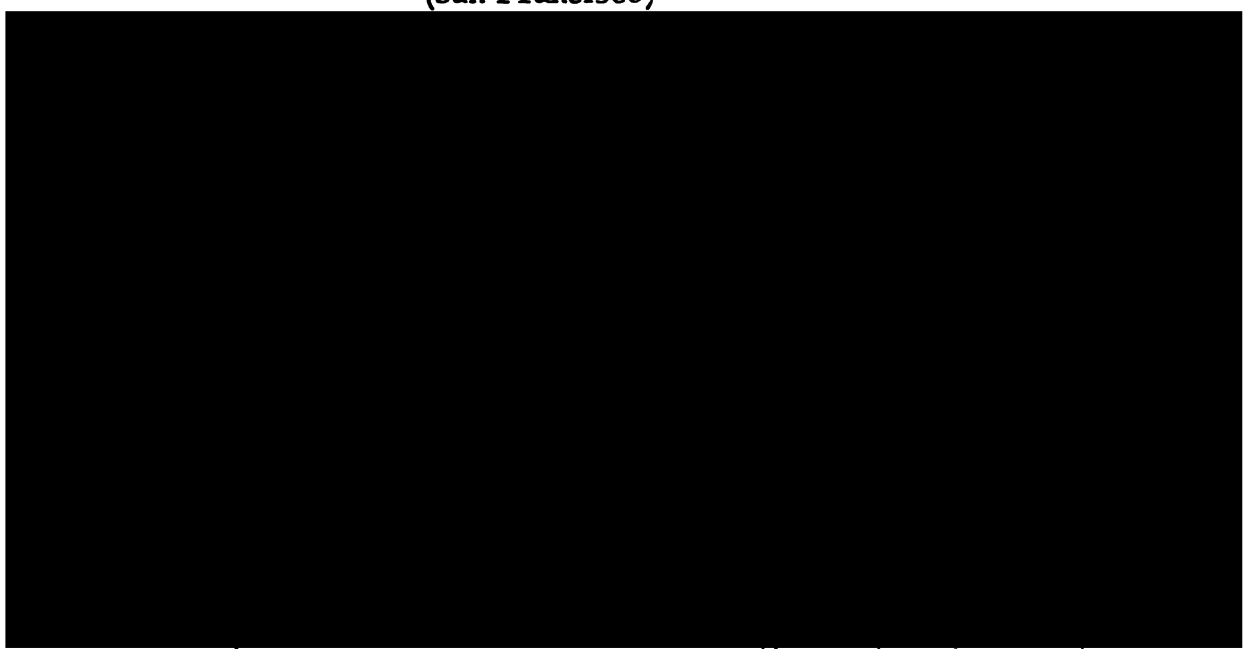
in

PHARMACOLOGY

in the

GRADUATE DIVISION

(San Francisco)



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Abstract

The 9, 14, and 18S forms of acetylcholinesterase (AChE) were solubilized from the main electric organ of Electrophorus electricus by extraction in phosphate buffer containing 1.0 M NaCl. Density gradient sedimentation studies in 5-20% sucrose containing various concentrations of NaCl indicated that the 14 and 18S forms were mostly aggregated below 375 mM NaCl but dis-aggregated at higher concentrations. The 9S form was not totally aggregated even in the absence of NaCl. The aggregation of the 14 and 18S forms was shown to be reversible by increasing the NaCl concentration to 375 mM or higher. The spontaneous autolytic conversion of the 9,14, and 18S forms (tailed molecules) to the 11.8S (no tail) form was shown to be accelerated by lowering the NaCl concentration.

The lack of form aggregation and conversion in 375 mM NaCl plus the binding characteristics of an Affi-202/N-methyl-3-aminopyridinium (MAP) affinity system resulted in the effective purification of the 14 and 18S forms. When these purified forms were separated by density gradient sedimentation, they were shown to have specific activities of about 460 mmoles acetylthiocholine/mg protein/hr., a 360 fold purification over the crude preparation. Polyacrylamide gel electrophoresis in SDS of the purified 14 and 18S forms established a minimum homogeneity of 96%.

The specific activities of the purified 14 and18S forms were used to calculate enzyme concentration in active site labelling experiments. By tritiated-DFP labelling and separation of labelled enzyme from unreacted labelling agent by Bio-Gel P-2 gel filtration, active site num-

bers of 4.2, 8.3, and 11.4 were calculated for the 11.8, 14, and 18S forms, respectively. These values are consistent with a model proposing that the 11.8, 14, and 18S forms are composed of 1, 2, and 3 tetramers of subunits, respectively.

Determination of bimolecular rate constants for the 9, 11.8, 14, and and 18S forms and various carbamate and organophosphate inhibitors revealed no substantial differences between the various enzyme forms. This result is consistent with electrophoretic evidence pointing to a common subunit for the various forms. The major difference between the 9, 14, and 18S forms on one hand and the 11.8 S form seems to be the presence of a tail-like structure in the 9,14, and 18S forms. Preliminary electrophoretic evidence pointed to a 110,000 MW protein as a candidate for the tail, while another 44,000 MW component may be a section of the tail.

To CHRIS ....

....Always on my mind

Table of Contents

Table of Contents (Continued)

Acknowledgements

I would like to give my deepest appreciation and warmest thanks to the following people:

Dr. Anthony Trevor for faith, patience, guidance and friendship

Dr. George Ellman for critical guidance in writing the dissertation and many hours of stimulating discussion over the years

Dr. Horace Loh for serving on both my orals and dissertation committees

Chuck Lee for incalculable help with illustration, instrument repair, animal preparations and for keeping things light

Leo Davidson for help with slides and photographs

Dr. Sin-Lam Chan for getting me started in the laboratory

Dr. G. Subrahmanyan for help with organic chemistry

Dr. Eugene Gardner for help with density gradient sedimentation and other tricks of the trade

My good friends Michael Gordon, Don Thorne, David Simpson, and Paul Fisher for being good friends

Jim Adams, Dr. Alvin Greenberg, Martha Harkey, Dennis Ward, and Dr. Wayne Settle for making the work days happy

Mom for being mom

Chris for help with everything

The department and all its members for the opportunity and the pleasure

List of Tables

List of Figures

Introduction

The Discovery of Cholinergic Neurotransmission

Even though Claude Bernard had observed the specialized effects of curare at the neuromuscular junction in the mid-nineteenth century, the concept of neurohumoral transmission was not formally presented until the early twentieth century. Langley (1901) observed that active agents of the suprarenal glands could mimic the effect seen when the nerve supply to certain muscles was electrically stimulated. In his classic work with adrenalin, Elliott (1905) established the hypothesis of neurohumoral transmission. He concluded that the action of adrenalin on smooth muscle was similar to the response seen following sympathetic excitation, and that the site of action was "at the junction of muscle and nerve." The implication clearly was that adrenalin or some similar substance was released by nerve and excited the muscle.

With the study of a powerful "muscarine-like" agent by Hunt and Taveau (1906), the identification of this factor as "Acetylcholine" by Ewins (1914), and the detailed studies of Dale (1914) on the esters of choline, the concept of neurohumoral transmission was extended to what became known as the parasympathetic nervous system. The existence of parasympathetic neurotransmission was further strengthened by the observations of Loewi (1921) in which a substance (Vagusstoff) released by vagal stimulation in one frog mimiced vagal stimulation when applied to another frog's heart. In 1929 Dale and Dudley established that acetylcholine (ACh) occurred naturally in animals. Following the work of Dale et al (1936), the concept of neurotransmission at the neuromuscular junction and in the parasympathetic nervous

system became virtually undeniable to most biological scientists. In this paper, Dale and co-workers showed that acetylcholine was released into the venous fluid upon excitation of the motor nerves to voluntary muscle. An interesting exception to the acceptance of the concept was Eccles (1937), who felt that the evidence was weak that ACh could be removed from the junction rapidly enough to account for known temporal aspects of transmission. Nevertheless, Eccles eventually accepted the hypothesis.

Identification of Acetylcholinesterase (AChE)

In 1914 Dale commented on the lability of acetylcholine and stated that ACh was probably hydrolyzed to its much weaker components, choline and acetic acid. He found that in alkaline solutions at room temperature and even in the cold, ACh quickly lost its activity. In addition, Dale surmised: "In the blood at body temperature it seems not improbable that an esterase contributes to the removal of the active ester from the circulation, and the restoration of the original conditions of sensitiveness." Stedman et al (1932) made the first contribution of evidence to this postulate by demonstrating that an enzyme in horse serum hydrolyzed choline esters faster than other esters. Accordingly, they used the term cholinesterase to describe this activity. Alles and Hawes (1940) demonstrated that two distinct types of esterase activity were present in blood. The enzyme in serum did not show an optimal substrate concentration and did not hydrolyze acetyl-β-methylcholine. On the other hand, the enzyme in red blood cells gave a distinct substrate optimum and hydrolyzed acetyl-β-methylcholine.

Nachmansohn and Rothenberg (1945) conducted a study in which the specificity of various esterases was tested. They concluded that for

cholinesterase, acetylcholine is hydrolyzed faster than any other substrate and that for other esterases, acetylcholine is not split at the highest rate. They also stated: "The esterase in all nerve tissues is either exclusively or predominantly cholinesterase." The enzyme in red blood cells and nerve and muscle fibers was named acetylcholinesterase by Augustinsson and Nachmansohn (1949) to describe the specificity toward ACh the enzyme showed. Additionally, AChE was not only present in muscle and nerve tissues, but in quite high concentrations that allowed the hydrolysis of large amounts of ACh (e.g. 1 g of innervated frog sartorius muscle split 8 mg of ACh per hour; Marney and Nachmansohn, 1938). These facts coupled with the existing role of ACh in nerve excitability, created the possibility that acetylcholinesterase was responsible for terminating the action of acetylcholine in excitable tissues.

Electric Fish and the Early Characterization of Acetylcholinesterase

Electric fish were known to be capable of generating bioelectric potentials at least as early as the eighteenth century (Reviewed in Nachmansohn and Neumann, 1975). Biological scientists have shown continual interest in the electric organs of these animals because of the magnitude of discharge (up to 600 volts in Electrophorus electricus) and recently because of the suspicion that these electrical potentials may be generated similarly to those known in muscle and nerve of other animals.

It is now known that the electric organ of Electrophorus electricus develops from embryonic muscle (Keynes, 1961). Electric organs are capable of generating electric potentials of great magnitude because of the arrangement of electric plates (electroplax) in the organ. The organ

consists of a stack of electroplax in series. This factor, in combination with the fact that only one surface of each plate is innervated, results in the summation of voltages when depolarization of innervated surfaces occurs (Keynes and Martins-Ferreira, 1953). It is known that the potential generated by an individual electroplax is similar to that generated by individual nerve or muscle cell; however, the organization of electroplax in series results in a significantly larger gross effect.

The study of acetylcholinesterase in electric organs began when Marney (1937) observed that the electric organ of Torpedo marmorata was capable of rapidly hydrolyzing extraordinary quantities of acetylcholine (2000-3000 mg ACh/g tissue/hour). In the following years, Nachmansohn and co-workers pursued studies involving the solubilization and initial characterization of AChE from electric organs. The quantity of AChE present in electric organs made them very useful for these studies. Also, the cholinergic nature of neurotransmission in electric organs had been established (Feldberg and Fessard, 1942). Thus, from a neurobiological standpoint, the study of AChE in electric organs seemed reasonable since the organs contained the full range of cholinergic components.

The initial purification of AChE was reported by Rothenberg and Nachmansohn (1947). By using toluene treated electric organ of Electrophorus electricus, ammonium sulfate precipitation, and ultracentrifugation, a 200-400 fold purification of the enzyme was achieved. In this procedure, AChE was solubilized by lengthy, autolytic release from the electric organ membranes. Soon to follow were a series of now classic studies that involved characterization of the fundamental kinetic

properties of AChE. Wilson and Bergmann (1950) and Wilson et al (1950) showed that the active site of AChE has two subsites, an esteratic site and an anionic site, and an acetyl derivative of the esteratic site is involved in the hydrolysis of ACh. A second step, de-acetylation is required for regeneration of the enzyme. Thermodynamic studies revealed that the de-acetylation step is rate limiting (for good substrates such as ACh) in the overall catalytic process (Wilson and Cabib, 1956).

Recent Studies of Acetylcholinesterase

The development of a chromatographic method for the purification of AChE (Kremzner and Wilson, 1963) resulted in highly purified enzyme. This approach represented a significant change in purification methodology. Up to this time all AChE purification procedures had utilized traditional, time consuming protein purification steps. However, the benzyldimethylaminoethyl cellulose resin used in this study was capable of rapid purification. This approach later evolved into the concept of affinity chromatography, which is widely used today. Kremzner and Wilson (1964) reported that the enzyme purified in this manner had a sedimentation value of 10.8S, a molecular weight (MW) of 250,000 and consisted of four active subunits. In contrast, Leuzinger et al (1969) presented evidence that the enzyme contained non-identical subunits, only two of which were catalytically active (Leuzinger, 1971).

Kalderon et al (1970) and Berman and Young (1971) introduced true affinity chromatography procedures capable of rapid and highly effective purification of the enzyme. The enzyme purified by these procedures was similar to the 250,000 MW(about 11S) form previoulsy mentioned.

However, in 1969 it became apparent that other molecular forms of electric eel AChE existed. This observation resulted from the discovery that most of the electric tissue AChE could be solubilized in pH 7 buffers containing 1.0 M NaCl. Using this extraction medium, Massoulie and Rieger (1969) demonstrated three molecular forms having sedimentation coefficients of approximately 8, 14, and 18S. In a subsequent study, Massoulie et al (1971) showed that these forms did not behave as typical globular proteins. For example, they were excluded from Sephadex G-200 and did not enter 7.5% acrylamide gels. On the other hand, the 11S form was known to be included in Sephadex G-200 and entered 7.5% acrylamide gels. Electron microscopic studies by Rieger et al (1973) and Dudai et al (1973) revealed that the 8, 14, and 18S forms appeared to be constructed of clusters of globular structures attached to long, filamentous "tail-like" structures. Of additional importance was the fact that the globular, 11S, form could be obtained from the tailed forms either by treatment with trypsin or through autolytic processes. This evidence led Cartaud et al (1975) to propose a physical model for the various forms. They suggested that the 11S form was a globular tetramer of subunits while the 8, 14, and 18S forms were composed of 1, 2, and 3 globular tetramers, respectively, linked to an elongated tail structure (see Figure 18, page 63).

Unfortunately, the tailed or asymmetric forms of the enzyme could not be easily purified by affinity procedures in use. Massoulie and Rieger (1969) had shown that the tailed forms aggregated in low salt buffers (10 mM NaCl); however, the affinity resins were unable to bind AChE if the ionic strength was raised enough to prevent aggregation. When purification was attempted in media low enough in salt to

permit binding, the aggregated forms were bound to the affinity resins in association with other proteins (Dudai et al, 1972a). The result was ineffective purification and low specific activities.

Design of Research

The difficulties encountered in purifying the tailed forms of AChE led to the development of "high" affinity approaches. Dudai et al (1972b) proposed the idea of using a ligand which permitted binding of AChE in buffers containing enough salt to prevent form aggregation. Thus, chromatography was attempted in 1.0 M NaCl. They synthesized an N-methylated acridinium derivative that showed extremely high potency (K_i = 0.3 uM) for AChE inhibition. Taking a different approach, Ashani and Wilson (1972) presented a novel covalent affinity resin for AChE purification. Their 2-aminoethyl-p-nitrophenyl methylphosphonate derivative of Sepharose bound AChE in high salt buffers and could be eluted with the cholinesterase reactivator, 2-PAM. These two approaches were, at least, partially effective, but each presented certain difficulties to investigators. For example, the synthesis of the acridinium ligand had to be modified by Dudai and Silman (1974), and not until recently was it successfully synthesized in another lab (Rosenberry and Richardson, 1977). The covalent resin was capable of binding other serine esterases as well as AChE and required the specialized synthesis of an oganophosphate AChE inhibitor.

Purification of the tailed forms of AChE was considered to be important for two fundamental reasons. First, AChE was known to be a membrane-bound enzyme. Since the "tail-like" structure seemed to be a candidate for a component that might bind AChE to the membrane, and since the globular form was obtained from the tailed forms by deg-

radative processes, it was suggested that the tailed forms were the native forms of the enzyme (Dudai et al, 1973; Rieger et al, 1973). The terms elongated, asymmetric, native, and tailed have all been used to describe these forms; for consistency in this presentation they will be called the tailed forms. Secondly, characterization of the tailed forms was incomplete. To pursue characterization studies such as active site determinations and analysis of the composition of the forms, required pure enzyme.

Active site studies with the tailed forms were also relevant to the characterization of the 11S, globular form of AChE. Although the active site stoichiometry of the globular form had been actively studied for many years, no definitive answer had appeared. Using direct counting of ^{3}H-diisopropylfluorophosphate labelled enzyme, Gordon et al (1976) found 2 active sites for the 11S molecule. Similarly, Leuzinger (1971) found 2 sites by diisopropylfluorophosphate titration of the active center. In contrast, when other titrating agents were used, 4 active centers per 11S molecule were found (Kremzner and Wilson, 1964; Rosenberry and Bernhard, 1971; Mooser et al, 1972). However, the same fluorescent titrator used in a study claiming 4 active centers, was used by Rosenberry et al (1972) to find only 3.3 catalytic sites. As previously mentioned, the 11S form could be obtained from the tailed forms by degradative processes (proteolysis or autolysis). Therefore, if the number of active sites for the tailed forms were to be compared to those found for the degraded form, a clearer understanding of these discrepancies should be obtained.

These factors, relating to the purification, active site stoichiometry and characterization of the tailed AChE forms, resulted in three main

objectives in the present research project. (1) The first major objective was to achieve an effective purification of the tailed forms of electric tissue AChE. In pursuit of this goal, it was proposed to study the salt concentrations that resulted in aggregation of the forms and/or conversion to the degraded 11S form. Next, the synthesis of affinity ligands which could purify enzyme under these conditions was to be attempted. Once the appropriate ligand and purification system was found it was hoped that the process could be refined such that the most convenient and effective purification could be consistently completed. Finally, it was planned to provide evidence for the purification by analyzing the homogeneity of purified preparations via sodium dodecyl sulfate/polyacrylamide gel electrophoresis. (2) The second major objective was to determine the number of active sites for the multiple enzyme forms. The goals in this section of the project were to understand the optimum methodologies required to accurately determine the numbers and to interpret the results obtained by these methods. (3) The final major objective was to conduct studies that would further characterize the tailed forms. Specifically, inhibitor sensitivity experiments and electrophoretic studies following manipulation of the forms with lytic enzymes were proposed.

Experimental Procedures

Synthesis of N-methyl-3-aminopyridinium iodide (MAP)

MAP was chosen as a potentially useful affinity ligand because of its structural similarity to standard AChE affinity ligands (Berman and Young, 1971; Rosenberry, et al 1972) and because it is easily synthesized. MAP was synthesized according to Goodkin and Howard (1974). 2g (0.021 moles) of 3-aminopyridine (Eastman Organic Chemicals) was stirred with 20 ml dry acetone and filtered to remove a small amount of undissolved solid. The volume was brought up to 30 ml with acetone and 6 ml (0.096 moles) of methyl iodide was added. The contents were stirred overnight at room temperature. The mixture was then filtered under suction, the solid was washed with acetone, and the product oven dried. The product melting point was 115-116° C. The inhibitory constant (k_i) for MAP was determined by the method of Dixon (1953). The The result of this experiment is shown in Figure 1.

Synthesis of Affinity Gels

MAP was covalently attached to the terminal carboxyl of Affi-Gel 201 or 202 (Bio-Rad Labs) by a standard water soluble carbodiimide coupling procedure. Affi-Gels are the gel filtration media, Bio-Gel A-15m, with an extender arm; 201 refers to the extender arm -O-$(CH_2)_3NHCO(CH_2)_2COOH$ while 202 refers to the extender arm -O-CH_2-$CONH(CH_2)_3NH(CH_2)_3NHCO(CH_2)_2COOH$. Each ml of gel was washed with water, suspended in 1 ml of water, and gently stirred with 750 u-moles EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), and the pH adjusted to 4.7. Then 100-200 umoles of MAP

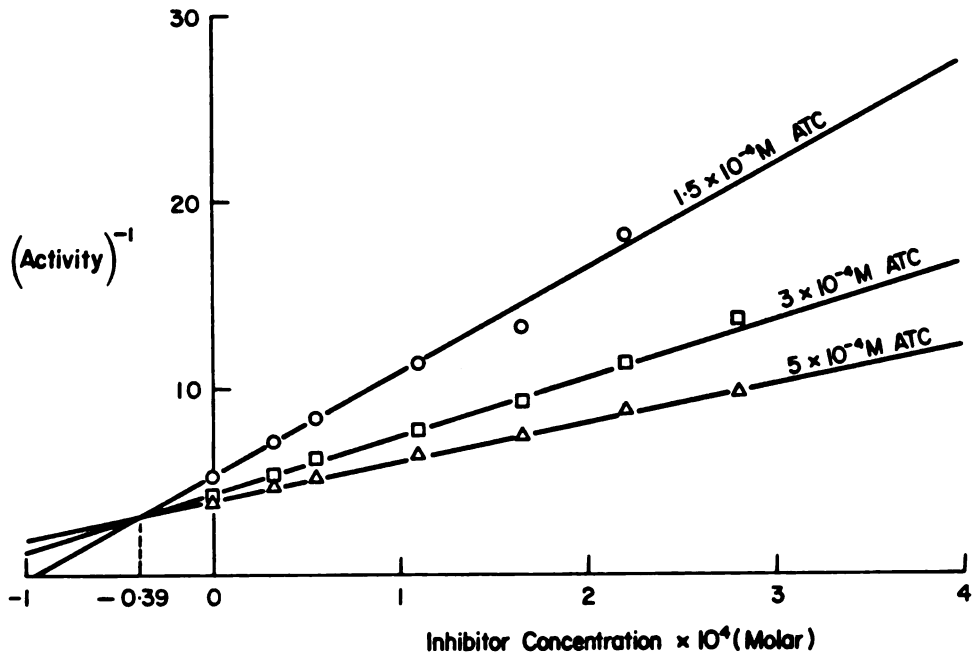

Figure 1. Inhibitory constant (Ki) for N-methyl-3-aminopyridinium iodide (MAP) as determined by the method of Dixon (1953). The enzyme used was from a commercial source (Sigma Chem. Co.) and was the globular (11.4S) form. ATC is acetylthiocholine. Activity is expressed as ΔOD 412nm/min.

were added, the suspension gently stirred, and the pH maintained at 4.7 with the addition of dilute HCl. The suspension was rocked overnight on a shaker at room temperature to assure that the reaction had gone to completion. Finally, the mixture was filtered and the solid washed with water. Some preparations of MAP were labelled with $^{14}C\ H\ I$ so that the number of ligand groups attached to the gel could be determined. Liquid scintillation counting indicated 0.8 umole and 1.3 umole of MAP present per ml of Affi-201 and 202 respectively. Figure 2 gives a condensed representation of the synthesis of MAP and its coupling to Affi-Gel 202.

Buffers

The buffers described in this section were prepared with dibasic sodium phosphate (J.T. Baker Chem. Co.), and were adjusted to pH 7.0 by the addition of 4N HCl.

Buffer A: 10 mM phosphate, 1.0 M NaCl
Buffer B: 10 mM phosphate, 375 mM NaCl
Buffer C: 10 mM phosphate, 100 mM NaCl
Buffer D: 10 mM phosphate

Solubilization of Acetylcholinesterase

AChE was solubilized from the main electric organ of Electrophorus electricus (obtained from World Wide Scientific Animals Inc.) by a modification of the procedure of Dudai et al (1972). All procedures were performed at 4°C. Fresh tissue was homogenized in 4 volumes of buffer A for one minute at speed 4 in a Sorvall Omni-Mixer followed by 10 strokes with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 78,000 X g for 80 minutes. The supernatant contained 65-75% of the total AChE activity. The crude supernatant was stored at 4° C for 2-3 months without loss of activity or conversion

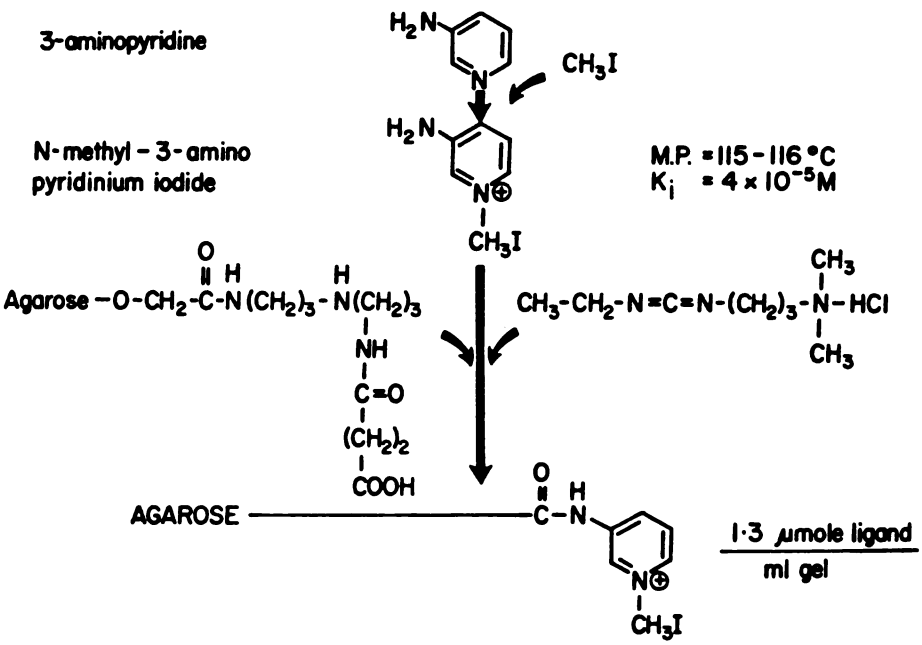

Figure 2. Synthesis of N-methyl-3-aminopyridinium iodide (MAP) and coupling to Affi-Gel 202. The synthesis and coupling are described in detail in Experimental Procedures.

to the globular (11.8 S) form of AChE.

Purification of Tailed AChE Forms

The procedure that gave the highest, most reproducible, purification will be described. The crude supernatant was dialyzed against 60 volumes of buffer B at 4 °C for 5 hours. The solution was then applied to the Affi-202/MAP gel at a flow rate of 10 ml per hour. Once loaded, the column was washed with 100 column volumes of buffer B and eluted in buffer B containing 20 mM decamethonium bromide (K& K Labs). The eluted fractions were dialyzed against 1000 volumes of buffer A for 5 hours. The entire procedure for extraction, purification and separation of AChE molecular forms is represented in Figure 3. To regenerate the affinity column, the gel was successively washed in buffer A containing 20 mM decamethonium bromide, buffer A, and buffer B.

Purification of Globular AChE

The 11.8 S form of AChE was purified in two ways. In both cases the crude supernatant used had been stored at 4° C for 3 to 9 months, resulting in the spontaneous conversion of all tailed enzyme to globular enzyme. In the first case, crude supernatant was dialyzed against 60 volumes of buffer C at 4° C for 5 hours. The solution was loaded on the Affi-202/MAP column, and the column was washed in buffer C and eluted in buffer C containing 20 mM decamethonium bromide. In the second case, crude supernatant was dialyzed against 60 volumes of buffer D at 4 °C for 5 hours. The solution was then applied to the Affi-201/MAP column; the column was washed in buffer D and eluted in buffer D containing 20 mM decamethonium bromide. In both cases, dialysis of eluted fractions was identical to that outlined for the tailed forms.

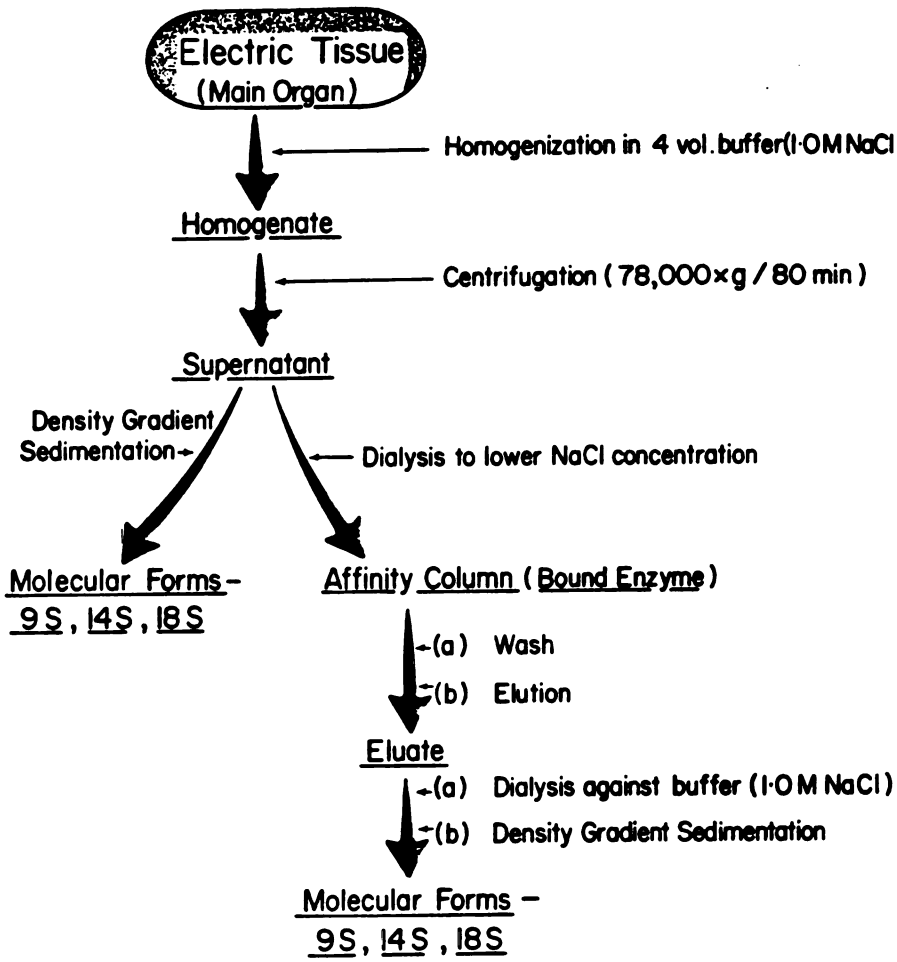

Figure 3. Procedural outline for extraction, purification and separation of molecular forms of AChE. The various procedures are given in detail in Experimental Procedures.

Density Gradient Sedimentation

The general procedure followed that of Martin and Ames (1961). 200 ul of protein solution was layered on 5.3 ml of a continuous gradient of 5-20% sucrose in buffer A at 4°C. The gradient tubes were centrifuged at 4 °C in a SW 65 rotor, Beckman L2-65 ultracentrifuge for 8 hours at 45,000 rpm or 5.5 hours at 55,000 rpm (g_{av}-hours = 1.16×10^6 in both cases). After centrifugation, the tubes were fractionated by puncturing the bottom of the tube and taking 10 drop (150 ul) fractions. A fractionation apparatus similar to that described by Martin and Ames (1961) was used. When purified enzyme was being used, the peak activity fractions from the affinity column were dialyzed as described above and concentrated to 150 ul by collodion bag vacuum filtration at 4 °C. The concentrate was removed, and the bag was rinsed with 50 ul of buffer A. The solutions were combined and layered on the sucrose gradient.

Aggregation Studies

In a series of studies designed to study the aggregation behavior of AChE forms, 1 ml aliquots of crude supernatant were dialyzed against 1 liter of buffer D containing either 0, 100, 250, 375, or 500 mM NaCl for 4 hours at 4° C. 200 ul of the dialyzed enzyme was layered on sucrose gradients containing either 0, 100, 250, 375, or 500 mM NaCl. The gradient tubes were then centrifuged and fractionated as described under Density Gradient Sedimentation.

Gel Filtration of Tailed Forms

At 4°C 1 ml of crude supernatant was layered on a 100 cm by 1.5 cm column containing Bio-Gel A 5m (Bio-Rad Labs) which had been previously equilibrated in buffer A. 1.5 ml fractions were collected

during elution of the column in buffer A. The void volume was estimated by determining the elution volume of Blue Dextran 2000 (O. D. 254 nm; Pharmacia Fine Chemicals technical information).

Enzyme Assays

Acetylcholinesterase activity was determined by the method of Ellman et al (1961). The 3 ml reaction mixture was maintained at 25 C° and contained 100 mM phosphate buffer, pH 8.0, 0.8 mM acetylthiocholine, and and 10 mM 5, 5-dithiobis-(2-nitrobenzoic acid). Catalase was assayed according to Beers and Sizer (1952). The reaction volume was 3 ml at 25°C and contained 50 mM phosphate buffer, pH 7.0 and 10 mM hydrogen peroxide. The time to give an O. D. 240nm decrease from 0.45 to 0.40 was measured. βgalactosidase activity was measured by the procedure of Craven et al (1965). The reaction mixture was 3 ml at 25° C and contained 100 mM phosphate buffer, pH 7.5, 1 mM $MgCl_2$, 100 mM mercaptoethanol, and 2.3 mM o-nitrophenyl-β-D-galactopyranoside (ONPG). The hydrolysis of ONPG was followed by monitoring the change in O. D. at 405nm.

Protein Assays

In crude preparations, protein concentration was measured by the method of Lowry et al (1951). In purified preparations, protein content was determined by the procedure of Bohlen et al (1973). In this procedure, primary amine groups of the protein are treated with fluorescamine (Fluram , Hoffman-La Roche) to produce a highly fluorescent derivative which is monitored spectrofluorometrically. 200 mM borate buffer, pH 7.0 was substituted for phosphate buffer to enhance the reproducibility of this assay.

Sodium Dodecyl Sulfate (SDS) - Polyacrylamide Gel Electrophoresis

The procedures followed the outline of Maizel (1971) while staining was by the method of Fairbanks (1971). The gels were 0.5 cm by 6.5 cm and contained 7.5% acrylamide and 0.1% SDS. The running buffer was 50 mM Tris, 400 mM glycine, pH 8.3. Samples were concentrated by colloidion bag vacuum filtration, mixed with an equal volume of solubilizing buffer (10% glycerol, 5% mercaptoethanol, 60 mM Tris, pH 6.7), and boiled in a capped tube for 5 minutes. After cooling, samples (10-150 ul) were layered on the gels and electrophoresed at 1.5 mAmps/gel for 2.5 to 3 hours. Bromophenol blue was used as the front running marker. After electrophoresis, the gels were removed by rimming and stained. Bovine serum albumin (BSA) was used to establish the sensitivity of protein staining, and BSA, ovalbumin, chymotrypsinogen, and trypsin were used as molecular weight calibration markers.

^{3}H -Diisopropylfluorophosphate (^{3}H -DFP) Purification Procedures

^{3}H -DFP (0.9 Ci/mmole; 1 mM; New England Nuclear) was purified according to Gordon et al (1976). It was found to be important to purify the compound the day preceeding use and to store the purified fractions at 4° C until use. Unless this precaution was taken, part of compound aggregated to a much higher molecular weight form. In this procedure, the crude ^{3}H -DFP was layered on a 60 cm by 0.6 cm column containing Sephadex G-10 (Pharmacia Fine Chemicals) and eluted in distilled water. Figure 4 shows the radioactivity profile observed following this procedure. Fractions 22-26 contained the anticholinesterase activity attributed to DFP, but only 43% of the radioactivity

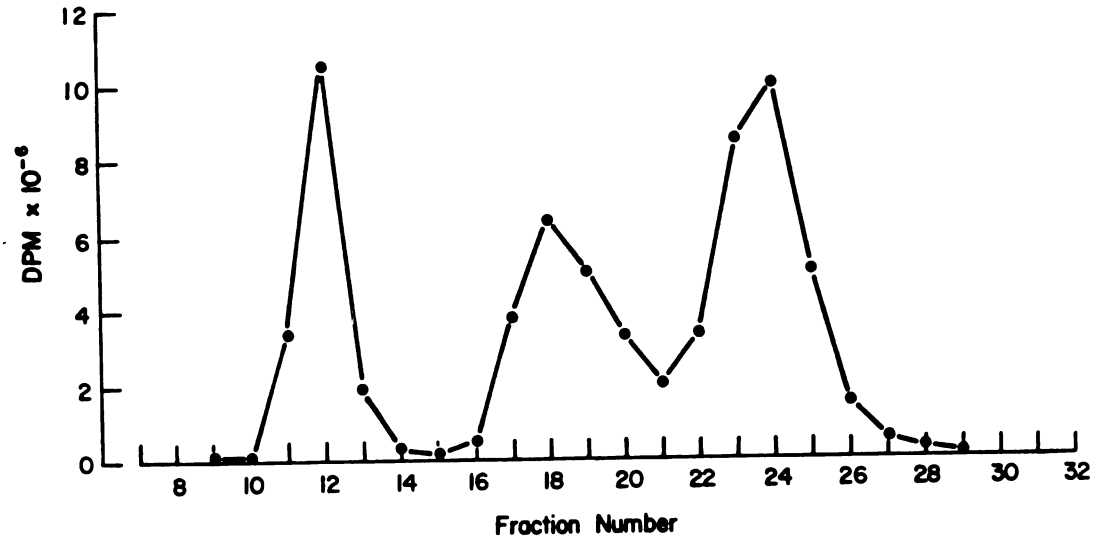

Figure 4. Purification of ^{3}H-DFP by Sephadex G-10 chromatography. The procedure followed the method of Gordon et al (1976). 30 ul of the crude compound was layered on the column, and eluted in water. Fraction volume was 600 ul. Fractions 22-26 contained the anticholinesterase activity while fractions 10-13 and 17-20 were radioactive contaminants.

applied to the column; thus, the manufacturer's radio-specific activity was adjusted from $2X10^{15}$ dpm/mole DFP to $8.5 X10^{14}$ dpm/mole DFP. Determination of the bimolecular rate constant for the material in fractions 22-26 verified the value ($1.7 X 10^{4}$ / M min) reported by Gordon et al (1976). This figure established that the concentration of DFP stated by the manufacturer was correct. The purified preparation was usable for 1-2 days before hydrolysis significantly reduced the DFP concentration.

^{3}H -DFP Labelling of AChE

100 ul of concentrated enzyme solution was mixed with 20 ul of crude supernatant (60 ug protein) at 4°C to insure the stability of the preparation. Control experiments had established that purified enzyme alone was not stable when brought to 25° C. The solution was then allowed to equilibrate at 25°C for 25 minutes. 100 ul of purified ^{3}H -DFP was added and the mixture gently swirled. This procedure gave final concentrations of DFP of $1X10^{-5}$ M and 500 mM NaCl in buffer D. To achieve 97-98% inhibition, the reaction required 35-40 minutes. Parallel controls containing water but no DFP were always run to insure that enzyme activity remained constant throughout the experiment and to determine enzyme concentration. Controls containing ^{3}H -DFP and crude protein but lacking enzyme were run in each set of experiments. Controls containing 1 mM edrophonium chloride (Hoffman-La Roche) in addition to the usual contents were also run to insure the specificity of the labelling procedure. Bergmann and Shimoni (1952)

demonstrated that quaternary, reversible inhibitors of AChE protect the enzyme against irreversible inhibition by organophosphates and carbamates. Thus, edrophonium chloride, one of the most specific reversible AChE inhibitors known, was chosen for the control experiments.

Separation of Labelled Enzyme from ^{3}H DFP

After labelling was completed, the reaction mixture was brought to 4 °C and loaded on a 0.6 by 60 cm Bio-Gel P-2 (Bio-Rad Labs) column equilibrated in buffer A. Taking 600 ul fractions, the labelled enzyme was eluted in fractions 11-13 in 5 hours. To assess the extent of recovery of enzyme, a series of enzyme activity and protein recovery experiments were run under identical conditions to those described for the labelling procedure except that ^{3}H -DFP was omitted.

Liquid Scintillation Counting

In all cases, 25-200 ul of sample was added to 10 ml of Aquasol (New England Nuclear). In the case of labelled enzyme, the volume of sample was fixed at 200 ul. Quenching was corrected for by the external standard channels ratio method. Chemiluminescence was detected when labelled enzyme was counted. This phenomenon resulted in a falling count rate for 8-12 hours after sample preparation. Stable counting was possible after 12 hours. All counting was done in Beckman LS-100C Liquid Scintillation Systems.

Determination of Biomolecular Rate Constants (K_i)

K_i was determined for a number of organophosphate and carbamate inhibitors of AChE and various forms of AChE by the procedure of Aldrich (1950). K_i is an overall acylation rate constant for the active

center of AChE and shows the following relationship to time (t), inhibitor concentration (i), and velocity (v) of substrate hydrolysis : $K_i = \frac{\Delta \ln v}{\Delta t\ i}$. This equation can be re-written as $K_i = \frac{1}{t\ i} \ln \frac{100}{b}$ where b = % of initial activity at (t). K_i was determined by taking the slope (m) of the line constructed by plotting log% activity against time. In this instance, $-K_i = 2.3\ m_i$. Where enzyme availability allowed, the average K_i determined at two or more inhibitor concentrations was reported (Main, 1964). The general procedure involved mixing 90 ul of enzyme solution with 10 ul of inhibitor solution and measuring the AChE activity of an aliquot at 0, 4, 8, 12, and 16 minutes. The 0 minute reading was taken from the control tube, which contained 10 ul water in place of 10 ul inhibitor solution. Under these conditions, the reaction contained 500 mM NaCl, 10 mM phosphate buffer, pH7.0 at 25° C. The conditions for enzyme assay have been mentioned previously.

Results

Gel Filtration and Sedimentation of Electric Tissue AChE Forms

AChE obtained from a commercial source (Sigma Chem. Co.) sedimented in sucrose density gradient centrifugation as shown in Figure 5. The single peak of activity was characteristic of these preparations, and the profile was not altered by the ionic strength of the sucrose gradient. Generally, proteins with an unknown sedimentation value can be assigned a sedimentation coefficient by comparison of the migration of the unknown with that of a standard protein having known sedimentation coefficient (Martin and Ames, 1961). See the Appendix for a detailed description of assigning S values. Catalase (11.4S) was the standard used throughout these studies, but another standard, β-galactosidase (15.9S), was also run occassionally to check the values obtained with catalase. The AChE in Figure 5 co-migrated with catalase and thus was assigned the sedimentation value of 11.4S.

The AChE solubilized by extraction of the electric organ in buffer A had a distinctly different sedimentation behavior. The sedimentation profile of AChE extracted under these conditions and sedimented in sucrose gradients that contained buffer A is depicted in Figure 6. Three peaks of activity with sedimentation values of about 9S, 14S, and 18S are seen. The relative abundance of these different forms was constant in 5 different eel preparations. If the salt extracted enzyme was allowed to stand for 3-9 months at 4 $^{\circ}$C before sedimentation, the sedimentation was similar to that observed for the commercial AChE preparation (Figure 5), but a sedimentation coefficient of 11.8S was obtained compared to the commercial preparation value of 11.4S. The

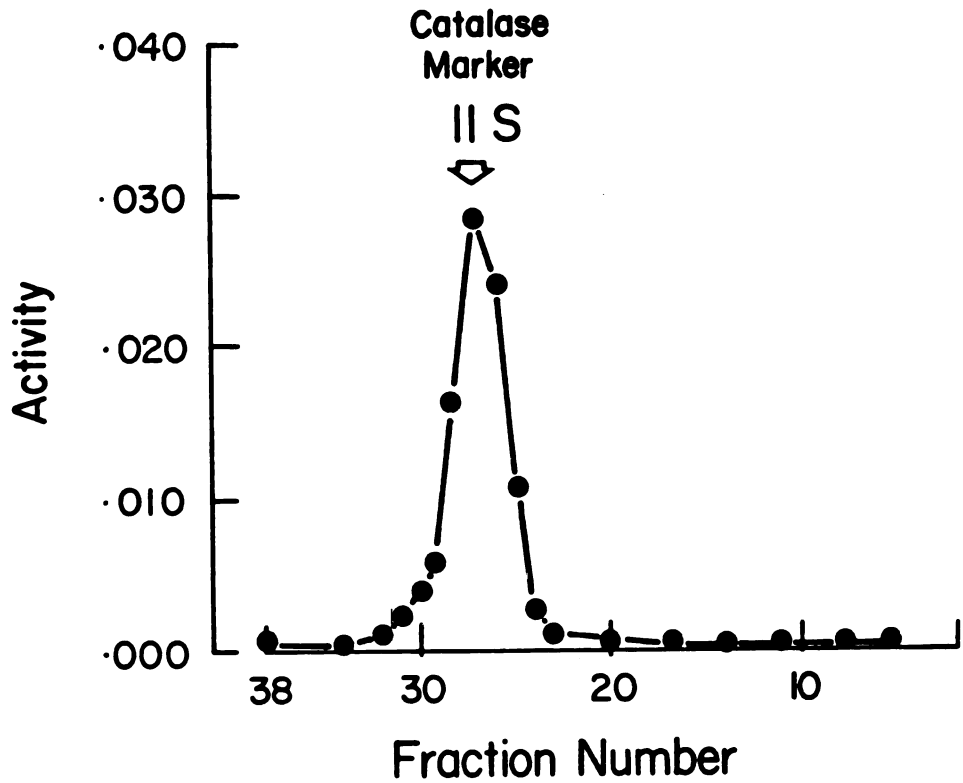

Figure 5. Sucrose density gradient sedimentation of a commercial preparation of AChE (Sigma Chem. Co.) in buffer A. AChE activity is expressed as ΔOD 412nm/min./ul.

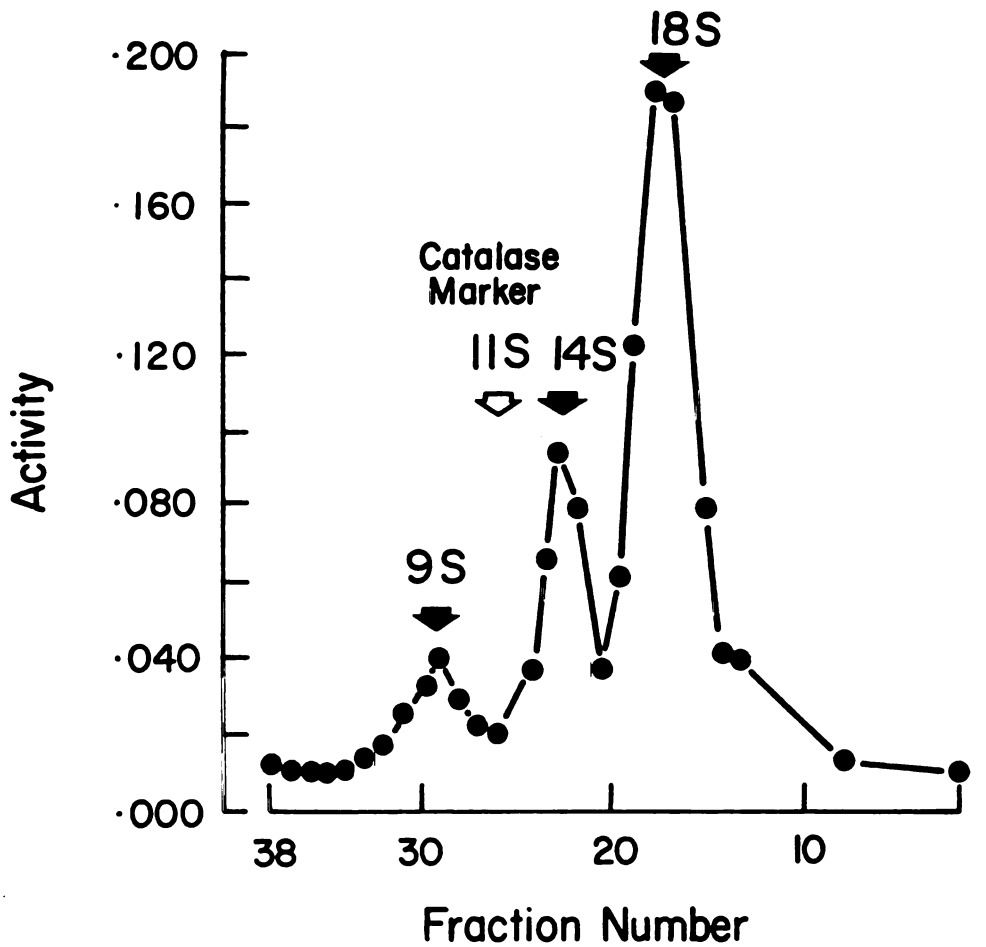

Figure 6. Sucrose density gradient sedimentation of crude AChE extracted from fresh electric organ in buffer A. AChE activity is expressed as ΔOD 412nm/min/30 ul.

sedimentation studies with the molecular forms obtained by salt extraction of fresh tissue are summarized in Table I. Also, included in the table are the sedimentation values for the commercially available preparation and the salt extracted forms after purification (the results of purification with Affi-202/MAP are described in the next section). As indicated by the standard errors, the values were consistent over a large number of determinations; this lack of variability was important because sedimentation behavior was the primary characteristic used to identify each form throughout these studies.

Although Massoulie and Rieger (1969) demonstrated that the tailed forms aggregated to rapidly sedimenting species in 10 mM NaCl and were not aggregated in 1.0 M NaCl, the exact concentration range required to cause aggregation has not been clearly appreciated. To discover these requirements and to test the reversibility of the aggregation process, a series of sedimentation experiments were performed. As outlined in the Experimental Procedures, samples of crude supernatant were dialyzed to various NaCl concentrations and sedimented in sucrose gradients containing the same concentration of NaCl that was present during dialysis. The sedimentation profiles for enzyme at 100 and 250 mM NaCl are given in Figure 7. In 100 mM NaCl, most of the activity was detected at the bottom of the gradient. The 14S and 18S forms were present in very small quantities; the 9S form, however, did not aggregate to the same extent as the 14S and 18S forms. When the experiment was conducted in 250 mM NaCl, the pattern was changed. Small amounts of the 14S and 18S forms appeared, although substantial activity was still represented by the aggregated form. Other forms, intermediate between totally aggregated species and the 18S

Table I

Sedimentation Coefficients of Various Molecular Forms of Electric Tissue AChE

Preparation	Sedimentation Coefficient (S) $\bar{X} \pm S_{\bar{x}}$	No. of Determinations
Crude Supernatant		
9S Peak	9.1 ± 0.1	10
14S Peak	13.9 ± 0.1	10
18S Peak	18.4 ± 0.2	10
Purified[a]		
14S Peak	14.1 ± 0.2	4
18S Peak	18.0 ± 0.3	4
Crude Supernatant (Converted)[b]	11.8 ± 0.2	5
Commercial	11.4, 11.4	2

Sedimentation coefficients were determined as described by Martin and Ames (1961). See the Appendix for details of this determination. $\bar{X}$ is the mean and $S_{\bar{x}}$ is the standard error of the mean.

a Purified enzyme was purified with the Affi-202/MAP system and the 14S and 18S forms were subsequently separated by density gradient sedimentation.

b Crude supernatant that is stored at 4°C converts from the tailed forms to the 11.8S globular form in 2-4 months by autolytic processes.

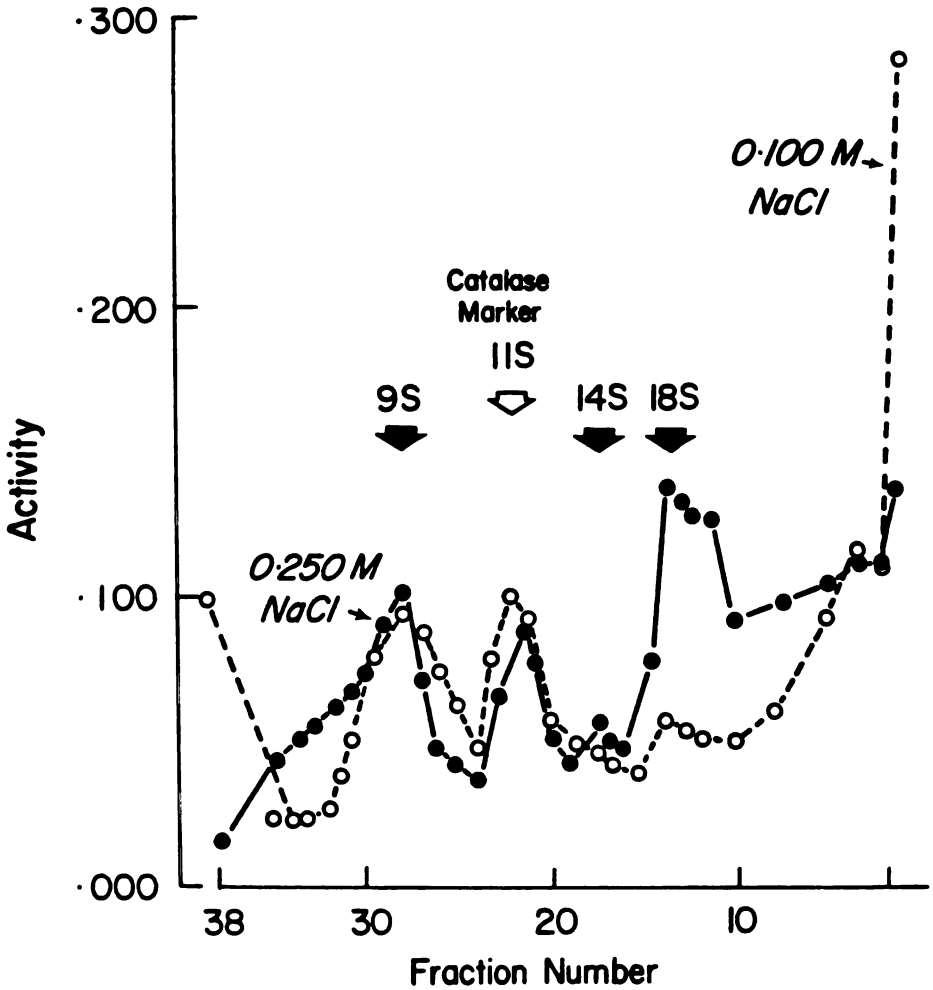

Figure 7. Sucrose density gradient sedimentation of tailed AChE forms in 100 mM and 250 NaCl. •- - -• are values for enzyme dialyzed and sedimented in 100 mM NaCl. •——• are values for enzyme dialyzed and sedimented in 250 mM NaCl. Enzyme activity is expressed as ΔOD 412 nm/min.

specie, also appear at this concentration. The results of two sets of experiments conducted in 375 mM or 500 mM NaCl are shown in Figure 8. At 375 mM NaCl the profile was essentially that of non-aggregated enzyme since only a small amount of activity appeared as the rapidly sedimenting aggregate. At 500 mM NaCl the forms were not aggregated.

If enzyme which had been dialyzed against buffer D and shown to contain mostly aggregates, was re-dialyzed against buffer A and sedimented in buffer A, the 9, 14, and 18S forms were detected and no aggregates were present. These studies indicated that the aggregation/dis-aggregation phenomenon is a reversible process which favors dis-aggregation at 375 mM NaCl and above. The 250 to 375 mM NaCl range is intermediate, neither aggregation or dis-aggregation being favored. Below 250 mM NaCl, aggregation is the predominant process.

Initially, it had been hoped to separate the tailed species of AChE by gel filtration as well as density gradient sedimentation. The result of running a sample of crude supernatant on Bio-Gel A-5m followed by elution in buffer A is shown in Figure 9. The three forms did not separate and were eluted jointly in the void volume. This result also illustrates the extreme asymmetry of these forms, as globular proteins with molecular weights of 5 million or less are included in this gel.

Purification of Tailed AChE Forms

9-aminopropylaminoacridine and N- methyl-5-aminoquinolinium iodide were synthesized in an attempt to provide high affinity ligands for the purification of tailed AChE forms in 1.0 M NaCl. At this stage of the project it was not appreciated that purification was possible at lower salt concentrations. Due to inadequate synthesis of ligands or

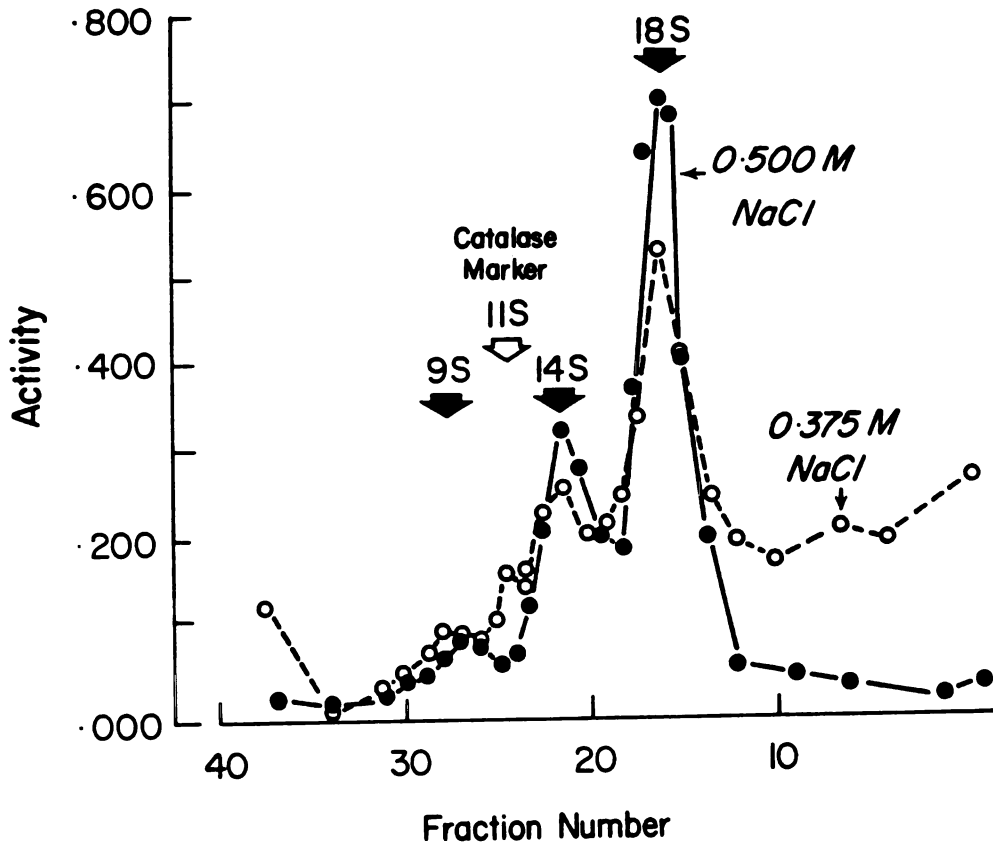

Figure 8. Sucrose density gradient sedimentation of tailed AChE forms in 375 mM and 500 mM NaCl. o---o are values for enzyme dialyzed and sedimented in 375 mM NaCl. •—• are values for enzyme dialyzed and sedimented in 500 mM NaCl. AChE activity is expressed as ΔOD 412nm/min.

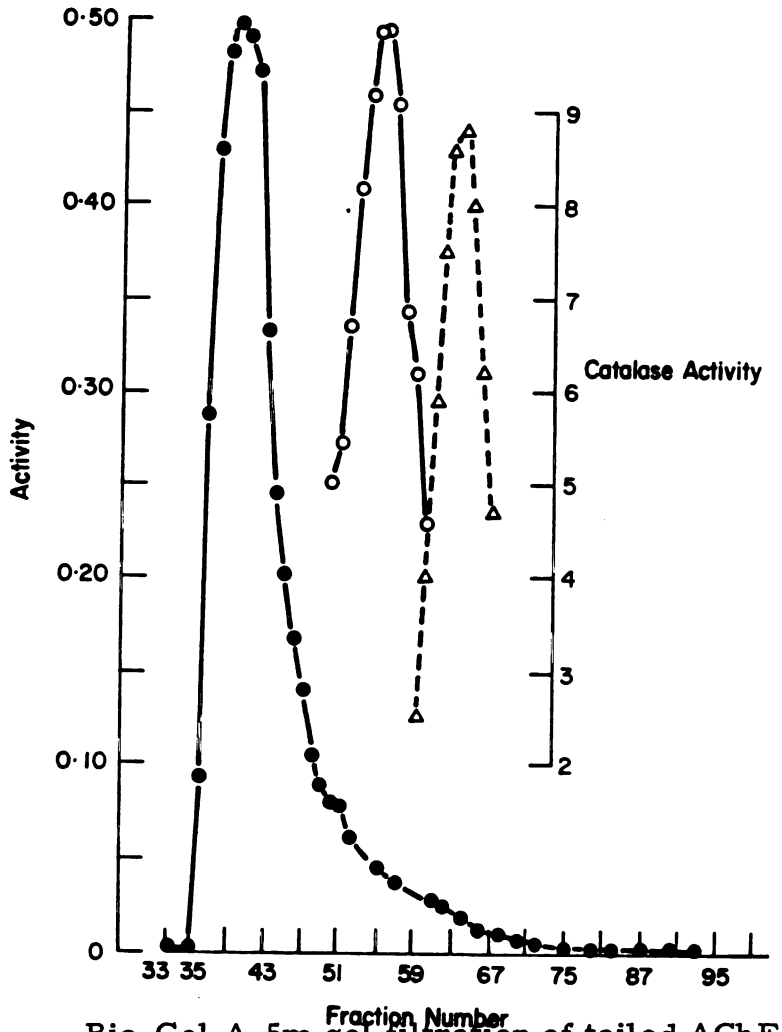

Figure 9. Bio-Gel A-5m gel filtration of tailed AChE forms in buffer A. ●—● is AChE activity expressed as ΔOD 412nm/min/25 ul. ○—○ is activity of the molecular weight standard, β-galactosidase (MW=595,000). ▲--▲ is activity of the molecular weight standard, catalase (MW=250,000).

inadequate attachment of the ligands to affinity gels, these ligands were abandoned (see Appendix for attempted syntheses). This difficulty in obtaining a high affinity ligand capable of binding AChE in 1.0 M NaCl resulted in attempts to develop purification schemes that utilized ligands which could be synthesized. The buffers used in these schemes either had no NaCl or used moderate NaCl concentrations (30 mM-500 mM) to enable the ligand to bind enzyme. Table II summarizes these studies. Three different column materials were tried: a trimethylammonium aniline (TMA) derivative of extended Affi-202 (Chan et al, 1972) and the Affi-201/MAP and Affi-202/MAP gels previously described. The TMA column bound the tailed forms in NaCl concentrations at least up to 500 mM. At lower NaCl concentrations (e.g. 150 mM) the procedure was complicated by conversion of the tailed forms to the globular form during enzyme preparation. To test whether the conversion was due to the chromatographic process or the NaCl concentration, aliquots of crude supernatant were dialyzed to less than 100 mM NaCl. After storage for 2-5 days at 4° C, the preparation was sedimented by density gradient centrifugation. The profiles were those of the globular (11.8S) enzyme. Thus lowering the NaCl concentration appears to accelerate the process of conversion of tailed to globular enzyme even in the absence of the gel.

Figure 10 demonstrates an interesting aspect of purification with the TMA column in 150 mM NaCl. If the column was eluted with 20 mM decamethonium bromide in 150 mM NaCl, the globular (11.8S) form was eluted almost exclusively. If this step was followed by elution with buffer A, all the tailed species were eluted. Unfortunately, the tailed species were not appreciably purified by this procedure. Nevertheless,

Table II

Purification Results in Preliminary Studies

Column	Loading NaCl	Loading %Bound	Elution Medium	Elution % Eluted	Forms Eluted	Specific Activity
TMA	150	98	D/20	29	11,14	51
			N/1	31	9,14,18	44
TMA	500	98	D/G	56	14,18	231
A1M	30	98	E/10	38	11	290
A2M	100	99	E/10	35	11	288

The experimental steps in each procedure followed the general outlines given in Experimental Procedures. Specific activities are mmoles ATC/mg protein/hr. Column is the affinity system used. TMA is a trimethylammonium aniline derivative of extended Affi-Gel 202. A1M is Affi-201/MAP. A2M is Affi-202/MAP. NaCl concentrations are in mM and are the conditions present throughout any one procedure unless specified otherwise. D/20 is 20mM decamethonium bromide. D/G is a gradient of 0-20mM decamethonium bromide. N/1 is 1.0M NaCl that followed D/20. E/10 is 10mM edrophonium chloride. Values for Forms Eluted are sedimentation coefficients (S).

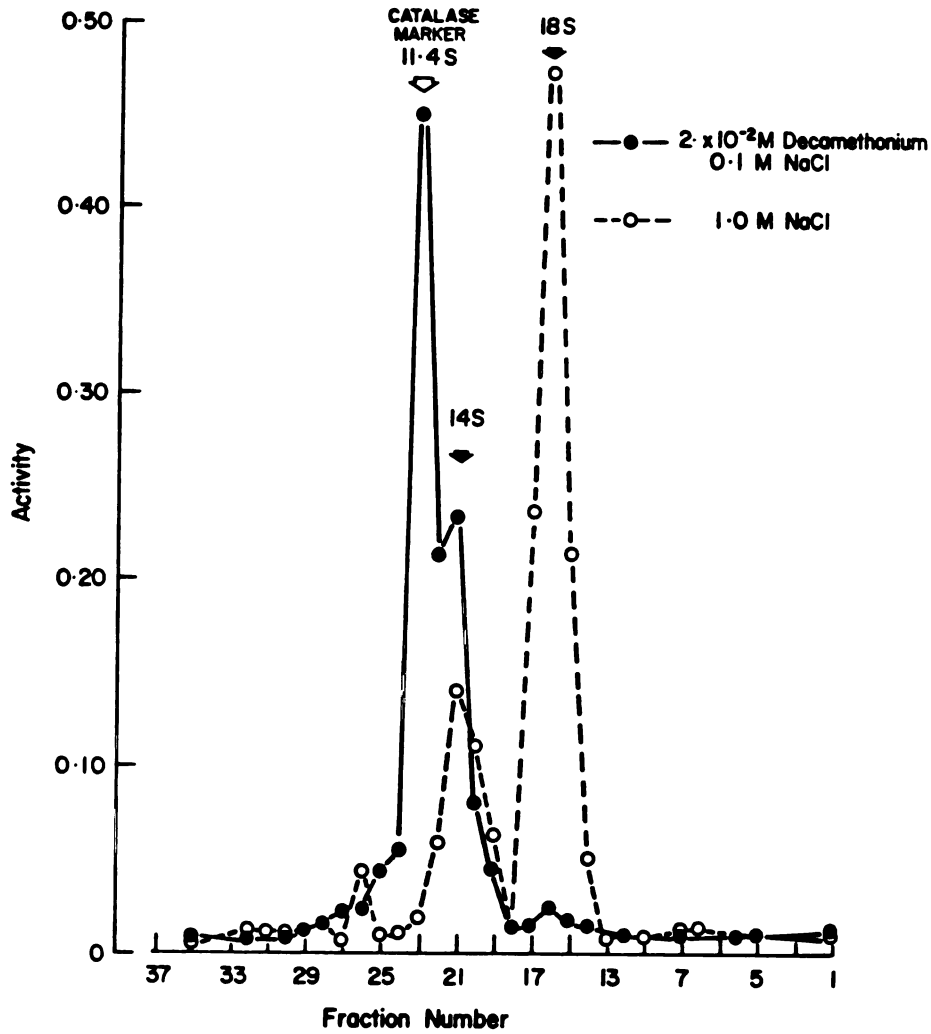

Figure 10. Sucrose density gradient sedimentation of AChE purified on the TMA column and eluted in 150 mM NaCl, 20 mM decamethonium bromide (•—•), or eluted in buffer A (▲--▲). Both gradients contained buffer A. AChE activity is ΔOD 412nm/min/10 ul.

the results were consistent with the concept that the binding of the globular enzyme (no tail) to the resin was of a different nature than the binding of the tailed species to the resin.

Running the TMA column in 500 mM NaCl, followed by elution in 20 mM decamethonium bromide in 500 mM NaCl resulted in elution of the tailed forms but at low specific activity. The specific activity could be raised sometimes by eluting the column in a gradient of decamethonium bromide (0-20 mM), but the results were not consistent enough to permit continued use of the procedure.

The Affi-201/MAP column bound AChE only if the NaCl concentration was below 100 mM. Typically, the column was run in buffer D; after washing and elution with 20 mM decamethonium bromide, enzyme of moderately high specific activity was obtained. Density gradient analysis permitted identification of this enzyme as the globular form. This result was consistent with the behavior of the tailed forms in low salt on the TMA column. That is, chromatography was not possible on any columns in low salt because of the conversion phenomena. Identical effects were observed using the Affi-202/MAP system in buffer C.

Fortunately, the Affi-202/MAP system bound the tailed species at higher NaCl concentrations. At 250 mM NaCl the tailed forms were bound to the column, but the density gradient profile of eluted enzyme was fragmented, with no particular form dominating. This effect might have been expected considering the intermediate forms observed in the aggregation studies at 250 mM NaCl. In buffer B the behavior of the Affi-202/MAP resin was markedly different. It showed high binding of the AChE activity applied, and a sharp peak of protein and enzyme activity was eluted with 20 mM decamethonium bromide in

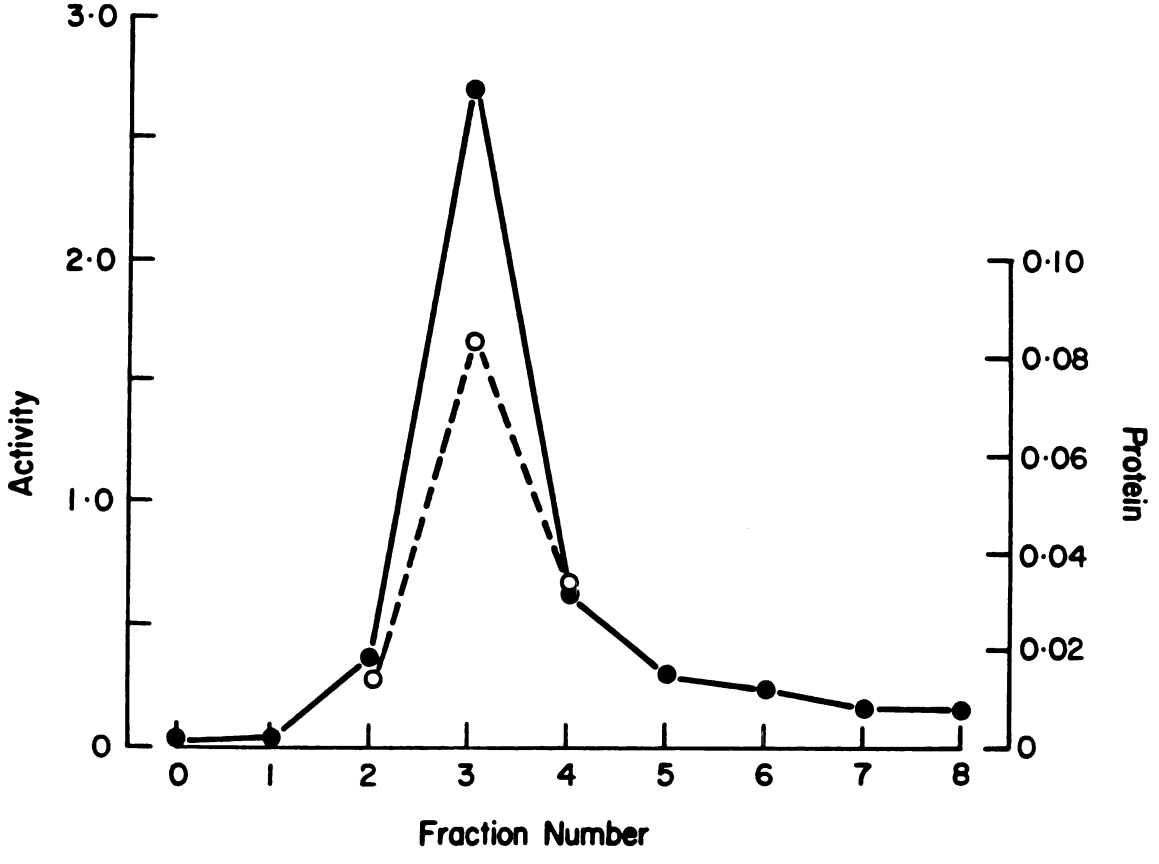

Figure 11. Elution of Affi-202/MAP in buffer B containing 20 mM decamethonium bromide. •—• is AChE activity (ΔOD 412 nm/min/ul) and o- - -o is protein (ug/ul) as determined by the fluorescamine procedure.

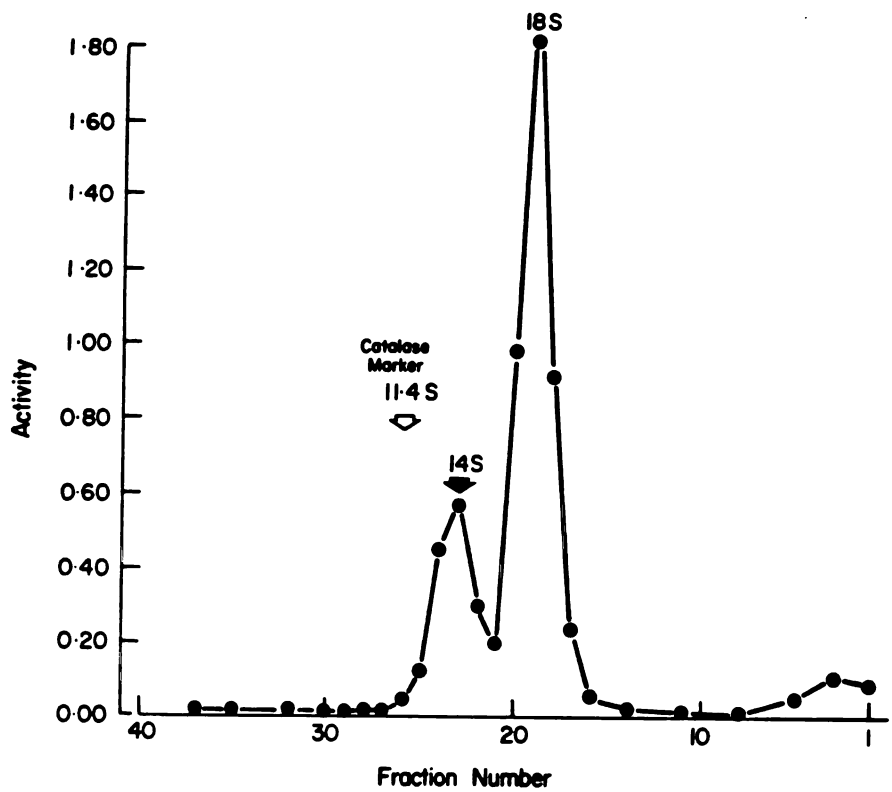

Figure 12. Sucrose density gradient sedimentation in buffer A of AChE eluted from Affi-202/MAP. The peak fraction of eluted activity was dialyzed and concentrated prior to centrifugation. AChE activity is ΔOD 412nm/min/ul.

buffer B (Figure 11). Figure 12 reveals that the 14S and 18S forms were present following dialysis and concentration of the eluted fractions. Table III summarizes the findings of a series of purification experiments with the Affi-202/MAP system. These results emphasize the reproducibility and effectiveness of the procedure. Since the specific activity of the crude supernatant was 1.2 mmoles acetylthiocholine hydrolyzed/mg protein/hour (mmoles ATC/mg protein/hr), the affinity peak represented enzyme that had been purified 240 fold. This factor was raised to 360 fold when the forms were subsequently separated on density gradient sedimentation.

Curiously, the 9S form of AChE did not appear in density gradient profiles of enzyme purified with the Affi-202/MAP system in buffer B. In an attempt to understand this problem, an analysis was made of the enzyme activity that was not bound to the column following loading. This enzyme was concentrated and sedimented by density gradient centrifugation. Analysis of the ratio of the 9S AChE to 14S and 18S before (Figure 6) and after (Figure 13) the column run indicated that 5 times more 9S form was present after chromatography. Apparently, the 9S form did not bind to the resin under these specific conditions. To test the role of MAP in binding the 14S and 18S forms, crude supernatant was dialyzed in buffer B as usual, but it was applied to a column containing only Affi-202 that had been equilibrated in buffer B. None of the enzyme activity was bound to the resin in this case. Thus, the presence of MAP on Affi-202 appeared to be a necessary condition for the binding of the 14S and 18S forms.

To establish the extent to which the 14S and 18S forms were homogeneous, the purified forms were subjected to SDS/polyacrylamide gel

Table III

Summary of Purification of the 14S and 18S Forms of AChE Using Affi-202/MAP and Density Gradient Sedimentation

Preparation	Specific Activity $\bar{X} \pm S_{\bar{x}}$	No. of Determinations
Crude Supernatant	1.2 ±0.1	3
Affinity (Elution Peak Fraction)	288± 44	10
14S Peak (Density Gradient)	458± 21	4
18S Peak (Density Gradient)	465± 20	5

The details of Affi-202/MAP chromatography and density gradient sedimentation are given in Experimental Procedures. Specific activities are mmoles ATC/mg protein/hr; $\bar{X}$ is the mean and $S_{\bar{x}}$ is the standard error of the mean. Typically, 19,800 ΔOD 412nm/min were applied to the Affi-202/MAP column; 84% of this activity was bound and 49% was eluted as a sharp peak of activity.

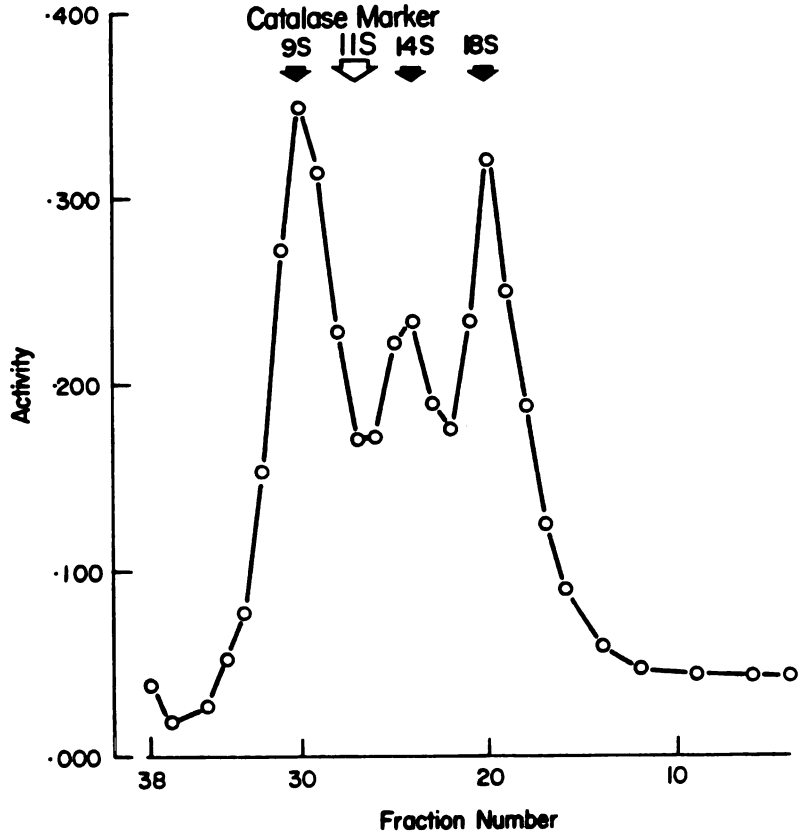

Figure 13. Sucrose density gradient sedimentation (in buffer A) of AChE that did not bind to Affi-202/MAP in buffer B. The unbound activity was concentrated prior to sedimentation. AChE activity is ΔOD 412nm/min/50 ul.

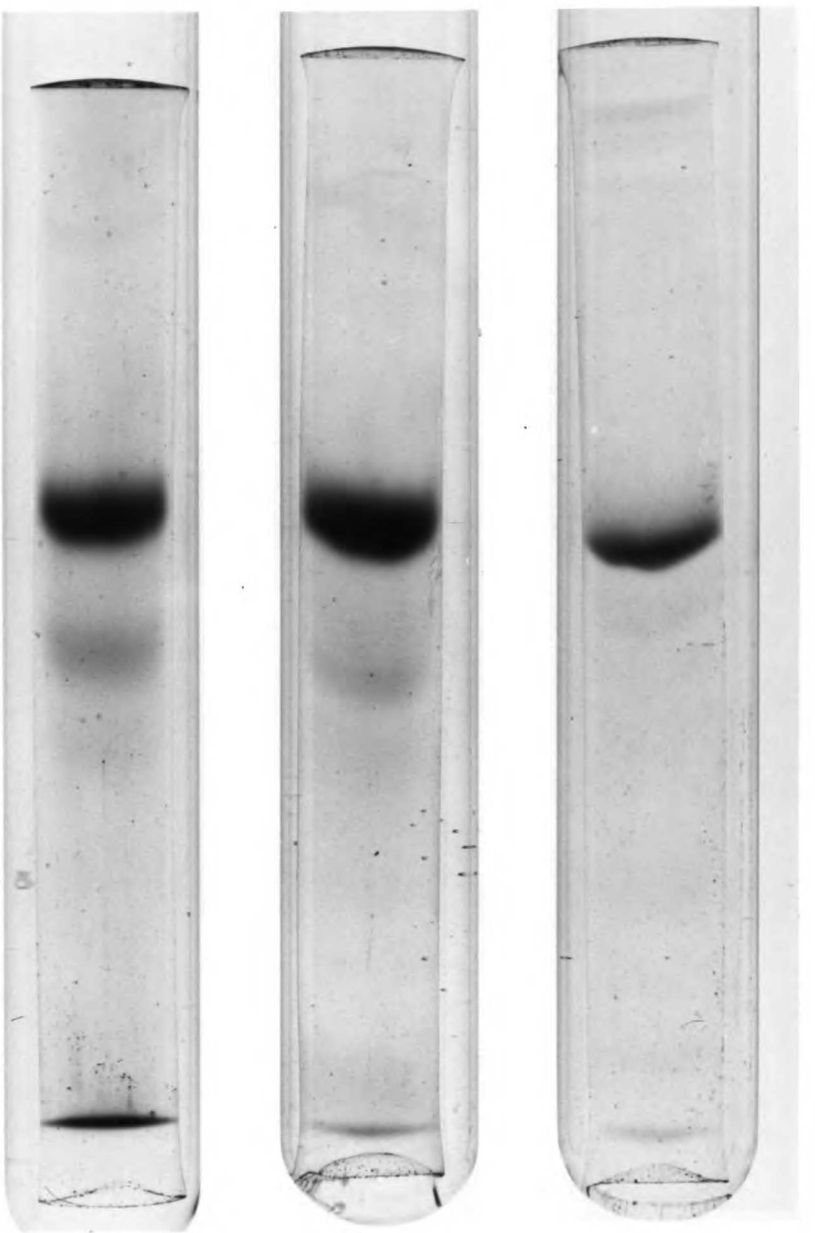

Figure 14. SDS/polyacrylamide gel electrophoresis of purified 14S and 18S AChE. Prior to electrophoresis the enzyme was reduced by boiling in mercaptoethanol. Migration is from top to bottom with the front running marker, bromophenol blue, appearing at the bottom. Left: 6 ug 14S AChE. Middle: 6 ug 18S AChE. Right: 2 ug bovine serum albumin (MW=68,000).

electrophoresis in the presence of mercaptoethanol. Figure 14 is an example of the results obtained when 6 ug of each purified form was electrophoresed. If recently prepared enzyme (1-2 weeks after sacrificing the eel) was used, one predominant band of 75,000 MW was observed. A faint band of about 55,000 MW was also visible. If enzyme older than 2 weeks was used, the 75,000 MW band was lighter, and the 55,000 MW band was darker. This process was progressive with the age of the preparation and indicated that the 75,000 MW protein was being converted to the 55,000 MW protein. These results were consistent with literature reports in which the 75,000 MW protein is considered to be the native subunit and the 55,000 MW protein a degradation product of the native subunit (Dudai et al 1972b; Rosenberry et al, 1974; Morrod et al, 1975; Bon and Massoulie, 1976). In some instances faint bands of approximately 110,000 MW and 44,000 MW were observed. It is possible that these proteins are minor contaminants, or that the band at 110,000 was the tail structure and the band at 44,000 was a section of the tail.

Most of the gels of purified forms showed 1 or 2 protein staining bands. Since the sensitivity of protein staining was found to be 0.1 ug, the homogeneity of the purified forms appeared to be at least 96%. It would have been of interest to use polyacrylamide gels lacking SDS and mercaptoethanol so that the gels could be stained for enzyme activity; this was not possible as the highly elongated tailed species will not enter acrylamide under non-denaturing conditions.

Active Site Determinations for the 14S, 18S, and 11.8 S Forms of AChE

The near purity of preparations of the 14S and 18S forms of AChE isolated by the Affi-202/MAP and density gradient procedures allowed

calculation of the molar concentration of the particular form in any experiment. To do this, the measured activity in the experiment was divided by the specific activity of the form in question (see Table III for the specific acitivity of purified forms). This calculation provided a measure of the amount (ug) of enzyme responsible for the observed activity. By using known molecular weight values for the AChE forms Dudai et al, 1973; Massoulie et al, 1975) the calculated amount was converted to moles of enzyme. The representative molecular weight values used for the 14S and 18S forms were 750,000 and 1,000,000, respectively. In the case of the 11.8S form, two experiments were conducted to provide a comparison of specific activity with known literature values. Using methods described earlier, the average specific activity of the 11.8S form was found to be 570 mmoles ATC/mg protein/hr. 320,000 was used as the molecular weight of the 11.8S form (Silman and Dudai, 1975; Bon et al, 1976). The molar concentration of the 11.8 S form was then calculated in all experiments using this information.

In determining the number of active sites of the AChE forms a known amount of enzyme was labelled with ^{3}H-DFP as outlined in the Experimental Procedures. Gel filtration was chosen as the method for separating labelled enzyme from unreacted label for the following reasons. If dialysis were to be used as a separatory method, the aging of phosphorylated AChE over the time (36 hours; Gordon et al, 1976) required for complete separation could confound the results. Colloidion bag vacuum filtration was also tested. However, preliminary experiments indicated that the tailed species were bound to the bags to some degree. This made quantitative recovery of labelled enzyme difficult.

Changes in the gel filtration/separation procedure of Gordon et al

(1976) were required to accomodate the tailed species of AChE. In preliminary studies the tailed species were shown to be retained in th gel when Sephadex G-10 and old preparations of Bio-Gel P-2 were used, even in high ionic strength buffers. For example, using the Bio-Gel P-2 preparation, purified preparations of 18S enzyme were loaded on the column and eluted in buffer A. Enzyme activity determinations of void volume fractions indicated that about 63% of applied enzyme activity was recovered. Simultaneous controls established that the drop in activity was related to some aspect of gel filtration (e.g. binding to the resin), rather than as a result of inactivation due to elution during the run. Table IV gives the results of a series of labelling experiments using this preparation of Bio-Gel P-2. Raw numbers and corrected active site numbers, taking into account the non-quantitative elution, are given. These results were not considered to be definitive; they are offered to give a rough idea of the magnitude of the active site numbers and as a comparison to later, more reliable results. For these results to be completely valid, a more thorough evaluation of enzyme recovery would have been needed.

In the course of further characterization of the elution of tailed species from Bio-Gel P-2, a pair of columns were made with another batch of Bio-Gel P-2. Surprisingly, this media gave quantitative elution of the tailed species. In experiments with the purified forms, 97% of enzyme activity and 86% of protein were recovered in the void volume. Likewise, experiments with the 11.8S form showed 105% of enzyme activity and 93% of protein recovered in the void volume. It should be noted that the 11.8S form gave quantitative elution on all gel filtration media used and at NaCl concentrations from 0-1.0 M.

Table IV

Summary of Preliminary Active Site Studies of the 14S and 18S Forms of AChE

Molecular Form	Moles AChE[a]	Active Sites/Molecule[b]		N
		Uncorrected	Corrected	
14S	4.5×10^{-12}	3.9	6.5	1
18S	2.1×10^{-12}	5.7	9.5	5

The studies were performed as described in Experimental Procedures.

[a] Moles AChE was the amount of enzyme present during labelling with ^{3}H-DFP.

[b] The preparation of Bio-Gel P-2 used did not show quantitative elution of AChE; an estimated correction factor was used to account for this (see Results).

It can be concluded from these experiments that the tailed forms were binding to the gels differently than the globular form of the enzyme. The differences between different batches of Bio-Gel P-2 remain to be explained. The elution of tailed forms of the enzyme on the preparation of Bio-Gel P-2 which gave quantitative recovery is shown in Figure 15. The elution of a void volume marker and purified ^{3}H-DFP is also included for comparison. It is important to note the complete separation of enzyme from label in this procedure.

The results obtained by labelling the 11.8S, 14S, and 18S forms with ^{3}H-DFP followed by gel filtration utilizing the quantitative preparation of Bio-Gel P-2 are given in Table V. To assure that the labelling with ^{3}H-DFP was directed toward the active site, a highly specific cholinesterase inhibitor, edrophonium chloride, was included in the labelling mixture of controls. In the presence of 0.001 M edrophonium chloride only 3-5% of the radioactivity present in the experimental case was recovered in the void volume. This suggested a high degree of specific active site directed labelling in the experimental case. The values in Table V have been corrected for this small amount of non-active site labelling. In early experiments, the void volume had been contaminated by high molecular weight radioactive contaminants. To check for this effect, control experiments that did not include enzyme were run for every batch of label that was purified. After observing that the purified label must be stored at 4° C overnight before use, the controls were negative in every instance. One additional control was tested. To assure that the enzyme taken from the density gradient separation of forms did not change to another form prior to labelling, a

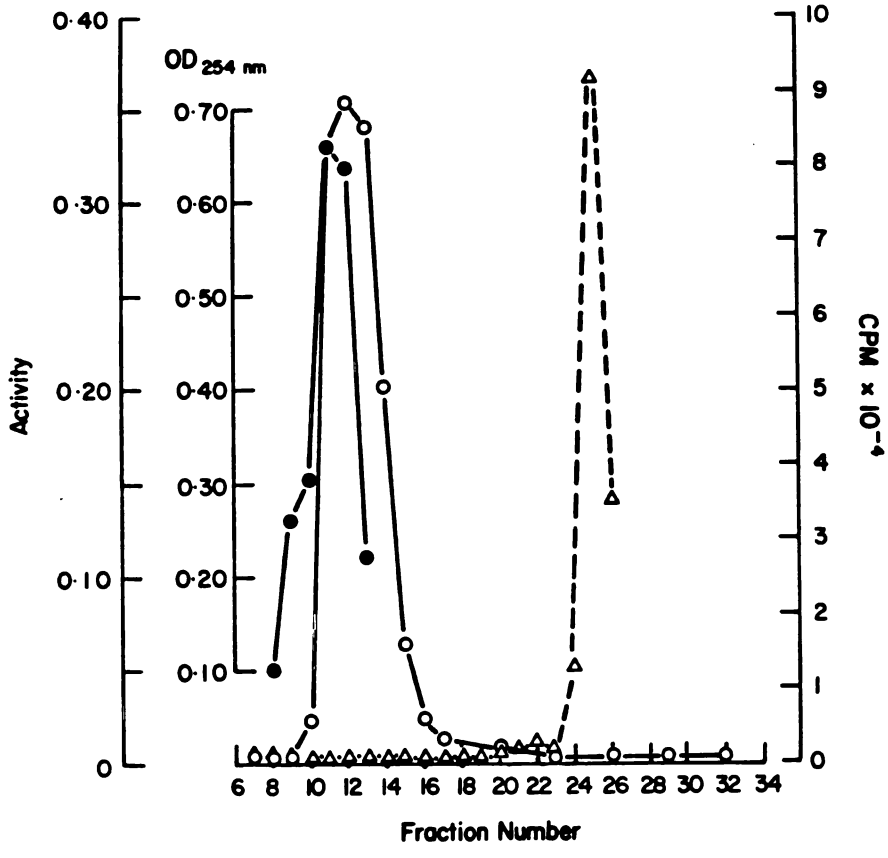

Figure 15. Bio-Gel P-2 gel filtration (in buffer A) of tailed AChE forms, purified ^{3}H-DFP, and blue dextran 2000. AChE activity is expressed as ΔOD 412nm/min/10ul •——•, blue dextran 2000 concentration is expressed as OD 254 nm •——•, and ^{3}H-DFP radioactivity is given in CPM▲--▲.

Table V

Active Site Numbers for the 11.8S, 14S, and 18S Forms of AChE

Molecular Form	Moles AChE[a]	Active Sites/Molecule[b] $\bar{X} \pm S_{\bar{X}}$	N
11.8S	$4\text{-}7 \times 10^{-11}$	4.2 ± 0.3	6
14S	$4\text{-}7 \times 10^{-12}$	8.3 ± 0.5	6
18S	$0.5\text{-}1 \times 10^{-11}$	11.4 ± 0.5	8

The studies were performed as described in Experimental Procedures.

[a] Moles AChE was the amount of enzyme present during labelling with ^{3}H-DFP.

[b] $\bar{X}$ is the mean and $S_{\bar{X}}$ the standard error of the mean.

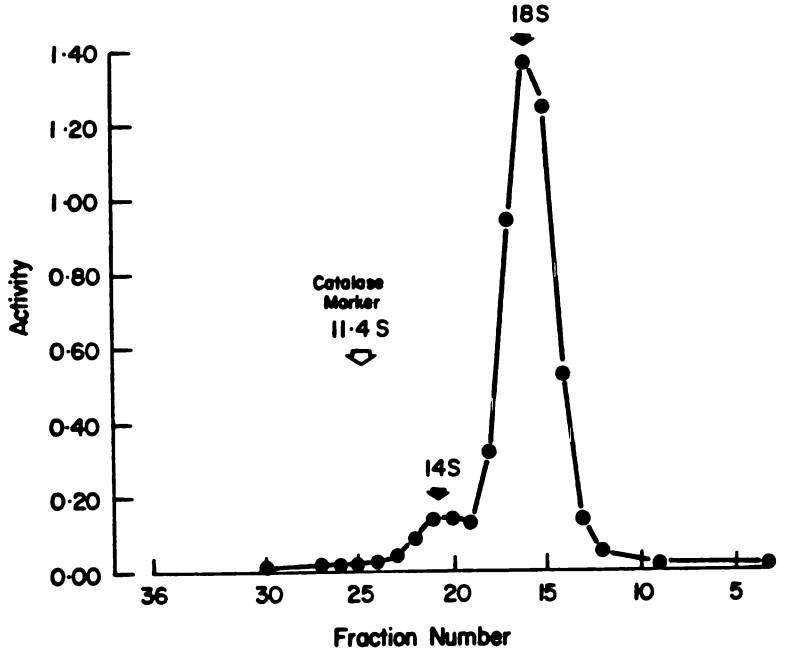

Figure 16. Sucrose density gradient sedimentation of 18S AChE in buffer A. The peak activity fraction of 18S enzyme from a density gradient was dialyzed to remove sucrose and re-sedimented. AChE activity is given as ΔOD 412 nm/ min/ul.

sample of 18S enzyme was re-sedimented. Figure 16 shows that the enzyme again sedimented as the 18S form. This control also suggested that the original separation of forms was not complete. Even when the peak fraction of 18S was selected, about 5% of the activity was the 14S form.

Bimolecular Rate Constants for the 11.8S, 9S, 14S, and 18S Forms

Bimolecular rate constants (K_i) for three esteratic site inhibitors of AChE and the available molecular forms of AChE were determined as described in Experimental Procedures. The three inhibitors were chosen to represent different classes of esteratic site inhibitors. Physostigmine salicylate is a highly potent carbamate; echothiopate iodide is a highly potent, positively charged organophosphate; dipropyl dichlorovinyl phosphate is a less potent, uncharged organophosphate. Table VI gives the bimolecular rate constant for each inhibitor with each molecular form. Figure 17 is a representative plot of Log% activity against time for the type of inhibition studied in this set of experiments. For each inhibitor, no obvious differences were seen for any of the molecular forms. These results can be interpreted to mean that the various molecular forms do not show any grossly different catalytic behavior. This is not to say that the forms lack kinetic differences of any kind. However, if kinetic differences do exist they must either be subtle or undetectable by this type of experiment.

Table VI

Bimolecular Rate Constants (Ki) for Various Inhibitors and the 11.8S, 9S, 14S, and 18S Forms of AChE

Molecular Form (S Value)	Physostigmine Salicylate	Echothiopate Iodide	DP-DCVP
	Ki X 10^{-6}	Ki X 10^{-6}	Ki X 10^{-5}
11.8	0.9	1.2	1.0
9	1.0	1.5	1.5
14	1.3	1.3	0.9
18	1.1	1.4	1.4

The experimental details and calculation of Ki are given in Experimental Procedures. The values for Physostigmine Salicylate are the average of determinations at two inhibitor concentrations. DP-DCVP is Dipropyl-dichlorovinyl phosphate (See Appendix for structure).

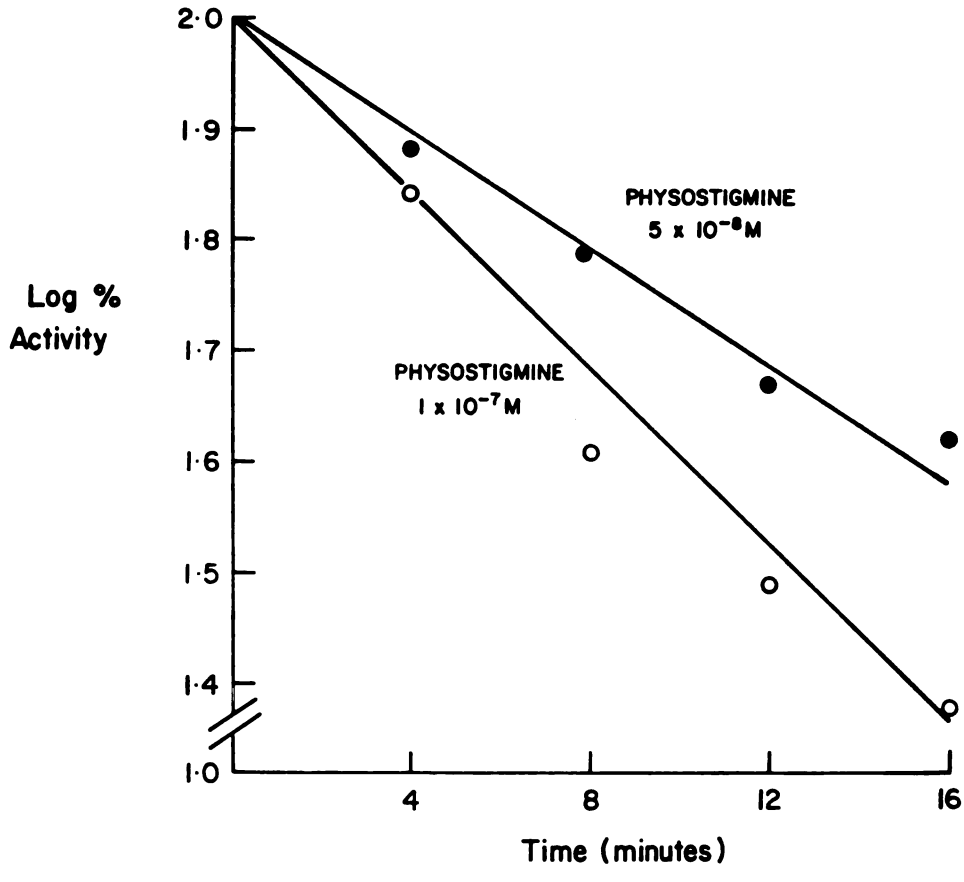

Figure 17. Determination of the bimolecular rate constant (Ki) for 18S AChE and physostigmine salicylate. 18S AChE and physostigmine salicylate were mixed at time zero; aliquots were removed at 4 minute intervals to determine residual AChE activity. Ki was calculated from the slope of the lines as discussed in Experimental Procedures.

Discussion

Aggregation Behavior of Electric Tissue AChE

The aggregation behavior of various forms of electric tissue AChE has not been clearly understood. Massoulie and Rieger (1969) showed that the tailed species of AChE become aggregated in 10 mM NaCl, but dis-aggregated in 1.0 M NaCl. In contrast, the 11S globular form of the enzyme did not aggregate at any salt concentration. However, whether the aggregation process was reversible or how various NaCl concentrations would affect the process was unknown. Some evidence existed pointing to reversibility of aggregation of certain species of electric tissue AChE. Grafius and Millar (1965, 1967) discovered polydisperse forms of AChE that aggregated in low salt but which were not aggregated in 300 mM NaCl at neutral pH. The confusing aspect of this work was that the preparation of AChE used typically gave 10.8S enzyme which did not aggregate (Lawler, 1959; Kremzner and Wilson, 1964). Consequently, the reversible aggregation behavior was largely ignored or considered to be anomolous.

The present study clearly indicated that the 14S and 18S forms of electric tissue AChE aggregate reversibly. Below 250 mM NaCl these forms were largely aggregated (Figure 7). Between 250 mM NaCl and 375 mM NaCl a complex pattern of aggregated, intermediate, and dis-aggregated forms was seen. Above 375 mM NaCl the forms were not aggregated (Figure 8).

The aggregation behavior of the 9S form was not accounted for as clearly. Unlike the 14S and 18S forms, the 9S form appeared to be dis-aggregated even in low salt. However, these studies were not

quantitative, and it cannot be concluded that the 9S form totally lacked aggregation behavior. A small fraction of the 9S form may have been aggregated while another fraction was not.

Since the 11.8S form did not aggregate while the 14S and 18S forms did aggregate, a good correlation exists between the presence of a tail structure in a particular form (14S, 18S) and aggregation behavior. Yet, how can the behavior of the 9S tailed form be accounted for? Possibly, the presence of a tail alone is not enough to account for aggregation. Either a particular tail structure is required or a particular combination of tail structure and other components is required for aggregation to occur. However, other factors may account for this discrepancy. Johnson et al (1977) presented evidence that what was usually called the 9S form was actually two forms unresolved by density gradient sedimentation. These two forms, a 7.5S form and a 9S form, were described as a globular dimer and a tailed tetramer, respectively. The globular form did not aggregate while the tailed form did.

If the tail structure is partially or fully responsible for the aggregation process, what is the molecular mechanism? The reversible, ionic strength dependent nature of the process points to the importance of ionic binding forces, at least in part. If ionic binding forces account for aggregation, then raising the ionic strength of the medium should interfere with the process. More complex relationships may be responsible for the process, though. If relatively hydrophobic regions of the enzyme are responsible for the binding that results in aggregation, raising the ionic strength of the medium could favor interaction of relatively hydrophilic regions of the enzyme with the solvent. At some

ionic strength, the equilibrium between dissolving and precipitating species should favor dissolution. The elucidation of the chemical composition of the tail and a detailed description of the molecular aggregate(s) will be required before the forces causing aggregation can be more fully understood.

The physiological significance of form aggregation is also not understood. Since the physiological medium is different than the in vitro solution, it cannot even be confidently stated that the forces responsible for the in vitro aggregation are operative in vivo. Nonetheless, the aggregation process has considerable significance to in vitro research. Since the aggregation is reversible in the range of 375 mM NaCl, purification studies are made possible that were not feasible at higher salt concentrations. Previously, 1.0 M NaCl was used to prevent aggregation. Fortunately, only one-third this concentration is actually required.

Purification of Native Forms of Electric Tissue AChE

In the past, purification of the native AChE forms has encountered a number of difficulties. Dudai et al (1972b) synthesized an N-methylated acridinium derivative that was capable of binding AChE in 1.0 M NaCl. However, attempts by a number of labs to replicate this synthesis were unsuccessful and even the modified synthesis (Dudai and Silman, 1974) was difficult or un-reproducible (Rosenberry and Richardson, 1977). Early attempts in the present study to synthesize acridine derivatives and other potentially high affinity ligands were also unsuccessful. Partially effective low affinity procedures in buffer lacking NaCl had been reported (Chen et al, 1973; Morrod et al, 1975). Attempts to use similar methods in this study were abandoned because the

the chromatography could not be conducted quickly enough (less than a day) to avoid the conversion of tailed species to the nontailed 11.8S form. The autolytic process that eventually converts all tailed forms to the globular form is accelerated in low ionic strength buffers. In addition, even when the tailed species were purified before conversion took place, the resulting specific activites were low. Therefore, neither high or low affinity ligands in high or low molarity NaCl were capable of reproducible, effective purification of native AChE forms.

The reversible aggregation behavior reported in this study raised the possibility that purification at intermediate NaCl concentrations is feasible. That is, by conducting affinity chromatography in the 250 to 375 mM NaCl range, the aggregation process could be avoided without resorting to higher NaCl concentrations that interfered with binding of the enzyme. In addition, the conversion of tailed species to globular species was inhibited at these ionic strengths. All of this was feasible provided the affinity system was capable of binding the enzyme at these ionic strengths. For affinity chromatography processes, 250 to 375 mM NaCl is still quite high (Dudai et al, 1972a).

The Affi-202/MAP column does bind the tailed forms in 375 mM M NaCl. Likewise, the column retained the enzyme during large volume washes, and released reasonable amounts of the purified 14S and 18S forms during elution (Table III, Figures 11 and 12). By following chromatography with density gradient sedimentation, the homogeneity of the individual forms was raised to 96% or higher (Figure 14).

The specific activites given here for the individual native forms are the first reported values. Dudai et al, (1972b) reported a specific act-

ivity of about 400 mmoles ACh/mg protein/hr. for purified but unseparated tailed forms. Recently, Massoulie and Bon (1976) reported a specific activity of 250 mmoles ATC/mg protein/hr., for purified but unseparated tailed forms. The general approach used by these workers to solubilize enzyme and conduct chromatography was similar to the approach employed in the present study, except that different affinity affinity resins were utilized. However, their hexylamido carboxyphenyldimethyethyl ammonium derivative of Sepharose 4B is much like Affi-202/MAP. Thus, the use of relatively low affinity ligands in moderate NaCl concentrations seems to be generally applicable to the purification of tailed AChE forms. The other reports of native form purification (Chen et al, 1973; Morrod et al, 1975) have not given specific activities. Additionally, none of the previous purification reports have given homogeneity data. The homogeneity information given here provides additional evidence that the enzymes purified by Affi-202/MAP chromatography and subsequent density gradient separation are relatively pure.

A distinct disadvantage of the Affi-202/MAP procedure is the inability of the system to bind the 9S tailed form (Figure 13). Complete characterization of the tailed forms is consequently not possible with this procedure. Whether this drawback is limited to the Affi-202 system is not clear. The density gradient profiles of purified forms from other procedures (Dudai et al, 1972b; Morrod et al, 1975) do not show 9S enzyme either. The state of affairs is further confused because Massoulie and Bon (1976) do not describe which purified molecular forms are obtained. It is possible that the anomolous behavior of the 9S form is related to characteristics of the molecule that also result in its special aggregation behavior.

The empirical factors discussed here point to the effectiveness of the Affi-202/MAP system in purifying the 14S and 18S forms of AChE. One of the more interesting aspects of this purification system is the evaluation of why the process works. Complex theoretical approaches have attempted to understand affinity chromatography in terms of the length of extender arm, the affinity of the ligand (inhibitory potency), and the number of ligand groups attached to the gel (Rosenberry, 1975). The emphasis is typically placed on the affinity of the ligand for the desired macromolecule.

However, the utility of the Affi-202/MAP system is not easily explained by the affinity of MAP for AChE. MAP has a Ki of 3.9×10^{-5} M which is over 100 times less potent than the acridinium ligand of Dudai et al, (1972b). Yet MAP binds enzyme in 375 mM NaCl while the acridinium compound binds enzyme in 1.0 M NaCl, only a three fold difference. Very similar ligands to MAP have not been able to bind AChE much above 100 mM NaCl (Dudai et al, 1972a; Morrod et al, 1975). Then why can the Affi-202/MAP resin bind enzyme at 375 mM NaCl? The answer appears to involve factors relating to the arm. The systems that failed to bind enzyme above 100 mM NaCl all employed extender arms of about 6-8 atom lengths. The Affi-201/MAP system has an extender arm of 8 atom lengths and will not bind AChE above 100 mM NaCl.

Thus, the length of the arm is quite important. However, it is not certain that arm length in the classic sense is the only important factor. The discovery of the importance of extending the ligand away from the gel matrix was made by Cuatrecasas et al (1968). The explanation given was that the binding site of the protein being purified

was not at the surface but buried inside the 3-dimensional structure of the protein. An extension of the ligand away from the matrix was required to reach this buried site. This concept may be valid; yet, there could be other explanations for the necessity of an extender arm. Although extender arms are viewed as being uninvolved in the actual binding process, evidence exists to the contrary in some cases. Massoulie and Bon (1976) demonstrated that the substitution of one arm for another of equal length but different chemical composition resulted in decreased binding of native AChE. In fact, they showed that the gel and original arm without added ligand bound tailed forms of AChE.

The Affi-202 resin itself did not bind the tailed forms unless MAP was attached. This does not say that Affi-202 does not interact or contribute to the overall binding process, however. In fact, evidence was presented that the tailed forms interacted with a number of media (colloidion bags, Sephadex G-10, Bio-Gel P-2). Thus it seems probable that Affi-202 does interact with the tailed forms of the enzyme.

O'Carra (1974) has proposed the term "compound chromatography" to describe affinity systems that seemingly are effective because of multiple factors. Thus, the affinity ligand, the composition and handling of the gel during preparation, the length and composition of the extender arm, the ionic medium, and the nature of the protein being purified must all be considered in evaluating an affinity system. It seems worth speculating that the effectiveness of the Affi-202/MAP system is related to such parameters. Contributions from MAP, the length and composition of the spacer arm and the composition of Affi-Gels, and the nature of the tailed forms must all be considered. It is worth noting that the globular and tailed forms showed differential

elution at 150 mM NaCl in the TMA system (Figure 10). A NaCl concentration may exist where the globular form will not bind to Affi-202/MAP while the tailed forms do. This would point to a special contribution of the character of the tailed forms to the functionality of Affi-202 /MAP. Regardless, in this special case and more generally in affinity purification, the process of affinity binding can no longer be treated solely as a simple, specific interaction of ligand and active site.

Active Site Stoichiometry of AChE Forms

By ^{3}H-DFP labelling and subsequent isolation of the labelled enzyme on Bio-Gel P-2 gel filtration, active site numbers of 4.2, 8.3, and 11.4 were found for the 11.8S, 14S, and 18S forms, respectively (Table V). These values suggest 4, 8, and 12 active sites per respective molecule.

The model of Cartaud et al (1975) proposes that the 11.8S specie is a globular tetramer of subunits, while the 9S, 14S, and 18S species are a tetramer, 2 tetramers, and 3 tetramers, in association with a long tail structure. The data of many workers point to a common active subunit for all species of electric tissue AChE (Dudai et al, 1972a, b; Rosenberry, 1974; Bon and Massoulie, 1976). Thus the Cartaud model predicts 4, 4, 8, and 12 active subunits for the 11.8S, 9S, 14S, and 18S forms, respectively. This is equivilent to predicting 4, 4, 8, and 12 active sites for the respective molecules. Although the data for the 9S form is missing, the results of the present study are highly supportive of this model.

The data also support the studies of the globular molecule (11.8S) which report 4 active sites (Kremzner and Wilson, 1964; Rosenberry

and Bernhard, 1971; Mooser et al, 1972). The studies reporting 3.3 sites (Rosenberry et al, 1972) and 2 active sites (Leuzinger, 1971; Gordon et al, 1976) are, however, at variance with these results. Even though methodological problems must always be considered as possible sources of error, the discrepancies being analyzed here are more likely related to calculational differences. Enzyme specific activity and molecular weight are parameters used to calculate the concentration of enzyme being labelled or titrated. In the past a variety of specific activities have been used and 250,000 was frequently used as the molecular weight of the globular form. Current evidence points to a value of approximately 600 mmoles substrate hydrolyzed /mg protein/hr. as being a generally acceptable figure for the specific activity of the globular form (Silman and Dudai, 1975). A value of about 320,000-330,000 also appears to be the currently recognized value for the molecular weight (Silman and Dudai, 1975; Bon et al, 1976). It is apparent that quite different final stoichiometries will be calculated if values other than these are used in determining enzyme concentration. Therefore, if the currently accepted specific activity and molecular weight values are correct, the globular enzyme has 4 catalytic sites. Values at variance with 4 can probably be adjusted to 4 by using the proper correction factor. Correction of this sort was used by Chen et al (1974) to correct a value of 3.3 active site per 11S molecule to 3.8.

Significance of Various Electric Tissue AChE Forms

The model discussed in the previous section is an important component of a larger picture that has developed as a result of work in a number of laboratories. The central point in this conceptual framework is the belief that a common AChE unit is combined in different

arrangements to form the various known molecular forms of AChE in electric tissue. It seems probable that the native form of the enzyme (the form that is present in the physiological state as a membrane bound protein) is the 18S form (Massoulie, et, 1971; Rosenberry and Richardson, 1977).

The 14S and 9S species may also be native forms, but they are probably partially degraded products of the 18S form. That is, the 14S molecule appears to be an 18S molecule in which one tetramer has been removed from the tail. Likewise, the 9S molecule has lost two tetramers. Nevertheless, the 18S molecule has not been extracted without the 14S and 9S molecules also being present. Until the form of the enzyme in situ is known, it cannot be stated with confidence that the 18S molecule is the native form. It is clear, though, that the 11.8S molecule is not a native form as it is a tailess tetramer, the tail having been removed by degradative processes. Figure 18 summarizes this information in pictorial form. In the figure, the active site information from this study has been integrated with information presented by Cartaud et al (1975). The active site information for the 9S form is speculative.

Two other molecular forms of electric tissue AChE have also been identified. One, which sediments in the range 7.4S to 7.7S appears to be a globular structure composed of two subunits. Therefore, it is referred to as a dimer or half tetramer (Massoulie et al, 1971; Millar et al, 1973). The second form, which has a MW of about 60,000 (Gordon et al, 1976) and which sediments at 5.3S is a completely active monomeric unit (Bon and Massoulie, 1976). Although the two molecular forms are present only in minute quantities, they are detectable under

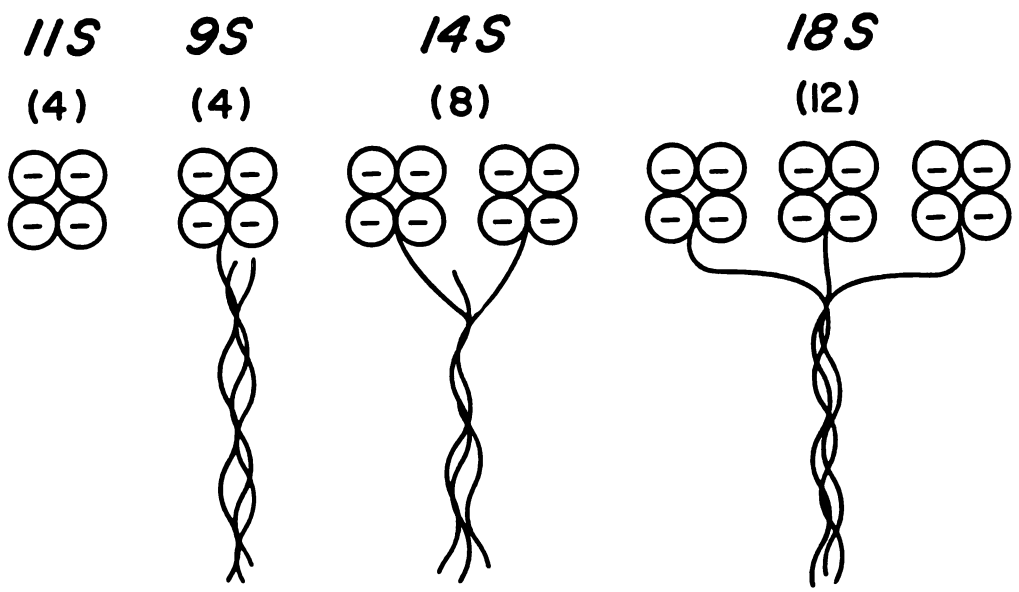

Figure 18. Summary of the molecular model and active site stoichiometry of the four predominant forms of electric tissue AChE. Active site numbers are in parentheses.

certain conditions, and they are thought to be further degradation products of the enzyme. Figure 19 gives a schematic view of how the different forms may be related to each other and, in this case, emphasizes some of the physical differences between the forms.

Electrophoretic evidence points to a basic subunit structure of 70,000 - 90,000 MW (Dudai et al, 1972a, b; Rosenberry et al, 1974; Bon and Massoulie, 1976). Other subunits have been observed (notably a 55,000 MW peptide), but they are known to be degradation products of the basic subunit (Rosenberry et al, 1974; Morrod et al, 1975). Analysis of available information leads to the conclusion that the basic subunit (or the 55,000 MW weight degradation product) and the monomeric molecular form are the same molecule. It is most significant that this molecule seems to possess the full range of catalytic activites attributable to the larger AChE forms. Since the various molecular forms are oligomers of the basic unit, it has been postulated that they all behave similarly with respect to catalysis of ACh. Evidence to support this speculation has been provided by Massoulie et al (1975); they showed that the Km's for the various molecular forms are identical.

The evidence presented in Table VI also tends to support the concept that the molecular forms are physically distinct, but catalytically similar. That is, in terms of overall catalytic events (e.g. inhibition resulting from reaction with active site inhibitors) the 11.8S, 9S, 14S, and 18S forms are indistinguishable. In the future, more discrete kinetic experiments may uncover differences between the forms. If such is the case, it will be intriguing to analyze if the differences are due to qualitatively different active site structures or to the spatial arrangement of active sites in the oligomers.

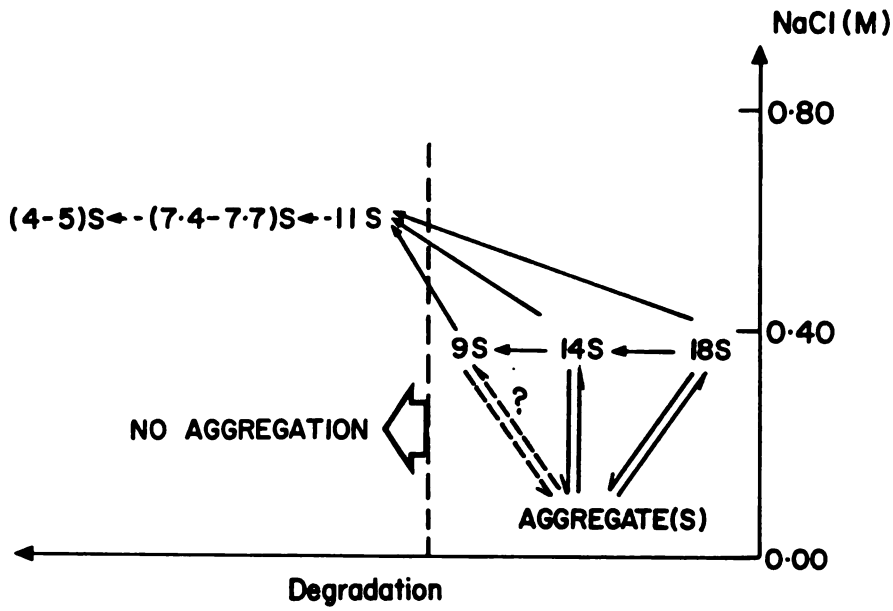

Figure 19. Schematic view of the relationship of all known molecular forms of electric tissue AChE.

The physical differences that are known between the forms are, at least in part, related to the presence of the tail structure in some of the forms. The tail has been elusive, and approaches to its isolation and characterization have been highly frustrating (Massoulie et al, 1975). Part of the problem may be that the composition of the tail is such that investigative procedures break it into fragments that are not identical from experiment to experiment. The present study has shown two electrophoretic components of 44,000 MW and 110,000 MW which are candidates for a section of the tail and the tail, respectively. A recent report by Rosenberry and Richardson (1977) has described a 40,000-44,000 MW electrophoretic component which may be a segment of the tail. The effective purification methods now available for tailed forms should lead to the discovery of the nature of the tail structure. Efforts along these lines promise to be one of the more exciting aspects of AChE research in the future.

Excitement about the tail results from the belief that the tail is responsible for anchoring the catalytic units of AChE in the biological membrane. It is interesting that tails of this sort are not limited to AChE. For instance, Porter (1977) has shown that one of the early components of complement, C1q, has a globular, tulip like, head attached to a collagenous tail structure. Recently, Lwebuga-Mukasa et al (1976) presented evidence that the tail of AChE from electric tissue of Torpedo californica is collagen like. These workers did amino acid studies which found residues in tailed species which were not found in the 11S, non-tailed form. These residues, hydroxyproline and hydroxy lysine are components of collagen. In addition, they were able to bind the tailed AChE forms to collagen rich membranes; non- tailed

forms did not bind. Johnson et al (1977) and Rosenberry and Richardson (1977) have presented preliminary evidence that the tails of AChE forms from Electrophorus electricus are collagen like.

Anatomical studies in Electrophorus electricus have utilized cytochemical stains for AChE to show inhibitor sensitive staining on the external innervated surface of the electroplaque, within organelles opening into the innervated surface, and in mucoid material extracellular to the innervated surface (Bloom and Barnett, 1966). The mucoid material is often referred to as basement membrane and is highly collagenous. It is possible that the tails of native enzyme bind to the basement membrane while the catalytic heads bind to other membrane components or are exposed to the extracellular fluid.

When future work clearly isolates and describes the tail structure, an important advance will have been made. Hopefully, such studies in conjunction with prevailing knowledge about solubilized molecular forms and studies with native membrane bound enzyme will lead to a better understanding of the role of AChE in cholinergic neurotransmission.

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Appendix I

Estimation of Sedimentation Coefficient (S) for Proteins

A detailed theoretical treatment of protein sedimentation behavior during constant velocity sedimentation is given by Martin and Ames (1961). The experimental utilization of these concepts will be outlined here. The density and viscosity characteristics of a linear 5-20% sucrose gradient are such that most proteins show a nearly constant rate of migration through the gradient. As a result, the relative distances travelled by different substances will be constant. Thus, if the migration of a highly characterized protein standard is compared with the migration of unknown proteins, S values can be assigned to the unknowns. A major assumption in this approach is that the various proteins involved have similar partial specific volumes. Martin and Ames (1961) assumed a partial specific volume of 0.725 ml/g for the proteins they studied; they calculated that such an assumption will introduce no more than 3% error in determining S values for most proteins. The partial specific volume for AChE has been determined to be 0.72 ml/g (Dudai et al, 1973) and 0.714 ml/g (Bon et al, 1976). Therefore, AChE meets this assumption.

In the present study the migration of different proteins was determined in the following way. The total number of fractions taken from the tube was divided into the total volume (5.5 ml) in the tube. This gave the volume of each fraction. From $V=\pi r^2 h$, h, the vertical height of a fraction was calculated. Then the distance from the midpoint of the top fraction to the midpoint of each successive fraction was determined by adding h to each successive value. Assuming that the sample

started migrating from the mid-point of the top fraction, this determination gave the total distance of migration for each protein. Then the ratio, R (distance of unknown migration/distance of standard migration), was calculated for the peak of activity of each protein. Because of the constant rate of movement of different proteins through this gradient, R= S unknown/ S standard. The well characterized enzyme, catalase (S=11.4), was used as the standard. Consequently, R X 11.4 = S unknown. Even though the number of fractions varied from about 36 to 38 for different fractionations, the sedimentation of the proteins studied here was quite constant (see Table I). In initial studies, the linearity of the gradients was checked by measuring the refractive index (% solids present) for fractions with a Valentine refractometer.

Appendix II

Synthesis of Other Potential Affinity Ligands

During the process of finding a suitable affinity system, the synthesis of a number of ligands was attempted. Attempts to utilize the products of these syntheses were unsuccessful, but the synthetic schemes and partial characterization of these compounds will be given here.

N-methyl 5-aminoquinolinium iodide (MAQ)

0.5g (0.0035 moles) of 5-aminoquinoline (Pfaltz and Bauer, Inc.) was added to 20 ml dichloromethane and 0.5 ml pyridine and stirred. 1 ml (0.007 moles) trifluoroacetic anhydride was added to the rapidly stirring solution. After 45 minutes, the dichloromethane was removed under vacuum, and the remaining solid was stirred with 50 ml of ether for 1 hour. The mixture was filtered, washed with ether, dried in a dessicator, and the solid was recrystallized from acetone/petroleum ether (1/4). 0.5g of the product, 5-trifluoroacetamido quinoline (MP= 144-147°C) was then refluxed with 3 ml acetone and 2.6 ml (0.042 moles) methyl iodide overnight. After cooling, the mixture was filtered, the solid washed with ether, and dried in a dessicator. The solid was recrystallized in methanol/acetone (1/9). 0.1g (0.0003 moles) of the product, N-methyl-5- trifluoroacetamido quinolinium iodide (MP= 220-222°C) was refluxed with 4 ml of methanolic HCl for 2 hours. After cooling, 50 ml ether was added, the mixture was filtered, and the solid washed with ether and dried. The product, MAQ, was linked to Affi-Gels 201 and 202, but the resulting affinity system did not bind AChE. Whether this deficiency was the result of insufficient ligand being linked to the gel or to inheritently poor affinity pot-

ential was not pursued. The structure and k_i of MAQ are shown in Figure 20.

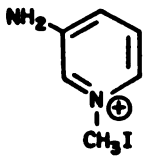
N-methyl-9-aminoacridinium iodide (MAA)

2g (0.01moles) of 9-aminoacridine (Aldrich Chem. Co.) was dissolved in 7.5 ml of methanol and refluxed with 2.6 ml (0.042 moles) ^{14}C-methyl iodide for 4 hours. The mixture was cooled, filtered, and the solid was washed with acetone. The product, MAA (MP=307-308 °C) was reacted with Affi-Gels 201 and 202 as described in Experimental Procedures. Liquid scintillation counting of the gel indicated that no ligand was attached. A check of the literature revealed that the amine function of N-methyl-9-aminoacridinium iodide is very unreactive due to the resonance tautomerism that occurs when the ring nitrogen is quaternized (Albert and Ritchie, 1942). In addition, the amine cannot be protected first and the ring nitrogen later quaternized as the ring nitrogen becomes unreactive. The structure and k for MAA are shown in Figure 20.

9-aminopropylamino acridine hydrochloride (APA)

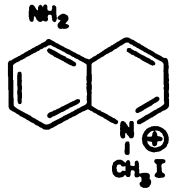
The synthetic procedure followed the outline of Albert (1966). 1g (0.005 moles) 9-chloroacridine (Eastman Organic Chemicals, 3.3g phenol, and 0.35 g (0.005 moles) 1,3-diaminopropane were mixed and heated for 1.5 hours at 120°C. The phenol was removed under reduced pressure. 50 ml of petroleum ether/ether (1/3) were added to the cooled resin that remained and the contents were stirred at 4°C for 6 hours. The mixture was filtered, washed with ether, and dried in a dessicator. Many variations were tried, but in all cases thin layer chromatography showed multiple products after the attempted synthesis even though the starting material, 9-chloroacridine, had only one

component. Dudai and Silman (1974) discovered that 1,2-diaminopropane must be used if side reactions are to be avoided. The structure of APA and the k for one of the purest preparations of APA are given in Figure 20.



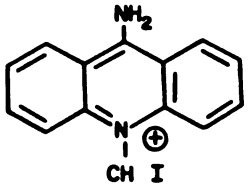
N-methyl-3-amino-pyridinium iodide

($K_i = 3.9 \times 10^{-5} M$)



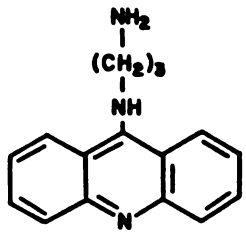
N-methyl-5-amino-quinolinium iodide

($K_i = 9.3 \times 10^{-6} M$)



N-methyl-9-amino-acridinium iodide

($K_i = 6 \times 10^{-8} M$)



9-aminopropylamino acridine

($K_i = 4 \times 10^{-7} M$)

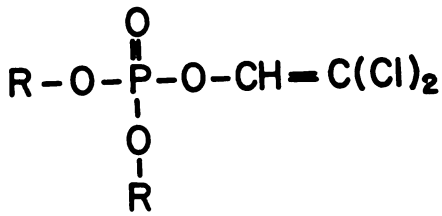
Figure 20. Structures and inhibitory constants (Ki) of four reversible inhibitors of AChE. The compounds were synthesized as described in the text. Ki was determined by the method of Dixon (1953) using a commercially available (Sigma Chem. Co.) source of 11.4S AChE.

Appendix III

Bimolecular Rate Constants for Bovine Brain AChE and Eel AChE

Various organophosphate, carbamate, and sulfonate inhibitors of AChE were used to determine the bimolecular rate constants (Ki) for a crude P_2 fraction (ammonium sulfate precipitate) of bovine brain AChE (Chan et al, 1972) and a commercial preparation of electric eel AChE (Sigma Chem Co.). The experiments were conducted as outlined in the Experimental Procedures. The general structural formulas for two especially interesting series of organophosphates are given in Figure 21. The phosphoric acid, 2,2-dichlorovinyl dialkyl esters (DCVP) were donated by Shell Development Co. The phosphoric acid, p-nitrophenyl dialkyl esters (PNPP) were the gift of Dr. T. R. Fukuto of the University of California, Riverside. The other inhibitors used were obtained from common commercial sources. The results with a mixed set of inhibitors are given in Table VII. The results with the DCVP esters and the PNPP esters are given in Table VIII. The ratios of Ki between brain AChE and eel AChE are large for some of the inhibitors; such differences are indicative of some basic difference in the catalytic behavior of the two enzymes. Although these studies do not pinpoint the differences mechanisticly, a number of general possibilites exist. For example, the actual catalytic apparatus may differ in the two cases or the environment of the catalytic site may vary for the two enzymes. It is also interesting to note that for the two series of organophosphates, the differences become larger as the alkyl substitution becomes larger.

Phosphoric acid, 2,2-dichlorovinyl dialkyl esters



Phosphoric acid, p-nitrophenyl dialkyl esters

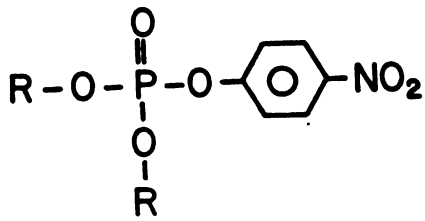


Figure 21. General structural formulas for two series of organophosphate AChE inhibitors. R represents various alkyl groups. For any individual compound R is the same in both positions.

Table VII

Bimolecular Rate Constants (Ki) for Various Inhibitors With Brain and Eel AChE

Inhibitor	Ki Eel AChE	Ki Brain AChE	B/E *
DFP	1.8×10^{4}	9.2×10^{4}	5.1
Methane Sulfonyl Fluoride	1.8×10^{2}	1.7×10^{2}	0.9
Phenylmethane Sulfonyl Flouride	0.9×10^{1}	5.0×10^{1}	5.6
Echothiopate Iodide	2.5×10^{6}	6.0×10^{5}	0.2
Neostigmine Bromide	1.1×10^{6}	4.7×10^{5}	0.4
Physostigmine Salicylate	1.2×10^{6}	3.6×10^{4}	0.3
1-Napthyl-N-methyl Carbamate	1.1×10^{5}	4.1×10^{4}	0.4
1-Napthyl-N,N-di methyl carbamate	1.0×10^{4}	7.1×10^{3}	0.7

The experiments were conducted as described in Experimental Procedures except that the concentration of NaCl in the incubation vessel was 100 mM. See the text of Appendix III for the sources of these two types of AChE.

* The ratio of Ki (Brain) to Ki (Eel)

Table VIII

Bimolecular Rate Constants (Ki) for Phosphoric Acid 2, 2- Dichlorovinyl Dialkyl Esters (DCVP) and Phosphoric Acid p-Nitrophenyl Dialkyl Esters (PNPP) With Brain and Eel AChE

Inhibitor/Alkyl Group	Ki Eel AChE	Ki Brain AChE	B/E *
DCVP/Methyl	5.0×10^{4}	4.3×10^{4}	0.9
DCVP/Ethyl	4.5×10^{4}	1.0×10^{5}	2.2
DCVP/Isopropyl	7.5×10^{2}	3.1×10^{4}	44
DCVP/Propyl	4.2×10^{4}	4.4×10^{5}	10.5
DCVP/n-Butyl	4.3×10^{5}	5.5×10^{6}	12.8
PNPP/Methyl	1.7×10^{5}	2.3×10^{5}	1.4
PNPP/Ethyl	1.7×10^{5}	5.5×10^{5}	3.2
PNPP/Isopropyl	1.8×10^{3}	2.8×10^{4}	15.6

The experiments were conducted as described in Experimental Procedures except that the concentration of NaCl in the incubation vessel was 100 mM. See the text of Appendix III for the sources of these two types of AChE. The general structural formulas for the DCVP and PNPP inhibitor series are given in Figure 21.

* The ratio of Ki (Brain) to Ki (Eel)