Purification of Acetylcholinesterase by Affinity Chromatography and Determination of Active Site Stoichiometry*

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SUMMARY

Specific inhibitors of acetylcholinesterase have been prepared and attached to Sepharose. When crude preparations of the enzyme are chromatographed on the affinity gel, the enzyme is selectively retained and may be eluted by a salt gradient. Conditions have been defined under which enzyme is purified some 20-fold in one step to a homogeneous and apparently pure species. An essential factor appears to be the distance the inhibitor is extended from the gel matrix by a chain of bridging atoms which form an attachment arm. A careful series of protein determinations was carried out to establish the ultraviolet extinction coefficient of the purified enzyme. Inherent differences among the protein values determined by various techniques appear to exist. They may in part account for discrepancies in values reported previously for the specific activity of homogeneous enzyme. With the use of an ε<sub>380nm</sub> = 18.0, a specific activity of 610 mmoles of acetylcholine hydrolyzed per hour per mg of protein was observed for the most highly purified enzyme. Active site titration measurements revealed 3.3 active sites per enzyme molecule.

Acetylcholinesterase (EC 3.1.1.7) was first isolated by extraction from the electric organ of Torpedo marmorata in 1938, following the discovery of an extraordinary concentration of the enzyme in this tissue (1). Purification of this enzyme from electric tissue of Electrophorus electricus by fractional precipitation of the enzyme at carefully controlled ammonium sulfate concentrations and pH values routinely gave a 100- to 200-fold increase in its specific activity<sup>1</sup> to a value of about 150, and further treatment by ultracentrifugation yielded a rapidly sedimenting, apparently homogeneous enzyme species which occasionally had a specific activity of 400 to 500 (2-4). Later investigations introduced multistep procedures using ion exchange and gel exclusion chromatography (5). These chromatographic procedures lead to the isolation of a homogeneous enzyme protein which has been crystallized (6), but the methods are laborious and result in an enzyme yield of less than 10%. In view of the difficulties in procuring electric tissue, such a yield is prohibitively small for use in studies aimed at elucidating the structure and properties of the enzyme. Moreover, the ultraviolet spectra and extinction coefficients reported for purified acetylcholinesterase (7, 8) are in disagreement. While this discrepancy may reflect a difference in purity of the two preparations, it seems likely that intrinsic differences in the quantitative protein determinations are also involved. The lack of a reference extinction coefficient for acetylcholinesterase has hampered the comparison of specific activities for purified enzyme with those reported in the literature (5, 6, 9). Extinction coefficients based on a variety of protein determinations will be presented in this paper, and hopefully they will facilitate the comparison of various enzyme preparations.

The technique of affinity chromatography was recently introduced in the purification of acetylcholinesterase (10) after it found wide application in a variety of protein purification systems (11). It involves the attachment of specific, reversible acetylcholinesterase inhibitors to a Sepharose resin matrix by means of the coupling agent cyanogen bromide. When a crude preparation of acetylcholinesterase is applied to a chromatographic column containing the modified resin, the acetylcholinesterase is selectively retained. The enzyme may be eluted from the column with a salt gradient. The extent to which the target protein is retained on the affinity column has been found to depend upon the length of the attachment arm which links the specific inhibitor to the resin (12). A number of specific inhibitors of acetylcholinesterase with attachment arms of varying length are currently under investigation in this laboratory as candidates for the affinity column purification of the enzyme. In this study we report the purifications of Electrophorus enzyme attained with the use of the inhibitor phenyltrimethylammonium attached to Sepharose 4B with arms of two lengths: mono-(6-
The chromatographic results obtained are compared directly with those from the conventional multistep procedure (5, 6). Although both procedures result in highly purified enzyme, the purification which can be obtained with affinity chromatography is slightly higher and the yield is much higher than that with the conventional procedure. During the course of this work a report on the purification of acetylcholinesterase by affinity chromatography appeared (9) in which a comparison of the retentive properties of several inhibitor-linked Agarose gels was made. The study presented here supplements that report in giving information both on somewhat different inhibitor ligands and on the elution profile and purity of the recovered enzyme. A high level of purification is achieved and a titration of enzyme-active sites is made to determine the number of active sites per molecule.

MATERIALS

Chromatographic Resins and Gels—DEAE-cellulose was obtained from Eastman; cellulose phosphate (Cellulofine-P) was purchased from Bio-Rad, Richmond, California; and Sephadex and Sepharose gels came from Pharmacia.

Titrating Agents—The synthesis of N-methyl-(7-dimethylcarbamoxy)-quinolinium iodide has been described earlier (13). 3,3'-Bis[α-(trimethylammonium)methyl]-azobenzene bromide; whose synthesis has been described (14), and 3,3'-bis[α-(14C)trimethylammonium)methyl]-azobenzene iodide, with a specific activity of 0.74 mCi per mmole, were generous gifts of Dr. N. H. Wassermann, Columbia University. For Bis-Q2 in water conditions under which the trans-isomer predominates to greater than 90% (14), Amax 316 nm (ε, 16,000).

Synthesis of Inhibitors

6-Carboxbenzoxaminocaproic Acid (I)—A solution of 39.4 g (0.3 mole) of 6-aminocaproic acid in 150 ml of 2 N NaOH was chilled to 0°. Carboxbenzoxyl chloride (57.5 g (95%), 0.32 mole) and 74 ml of 4 N NaOH solution were added simultaneously with good stirring over a period of 50 min, maintaining the reaction temperature 0-7°. After stirring an additional 30 min, the reaction mixture was extracted three times with about 100 ml of ether to remove excess carboxbenzoxyl chloride, and the aqueous solution was acidified slowly to pH 3 with 6 N HCl. The product was extracted three times with 250 ml of ether, and the combined ether layers were washed with brine solution and dried over anhydrous Na2SO4. Evaporation of the ether afforded an oil which was crystallized from benzene and a small amount of hexane on chilling in a refrigerator overnight. The crude product was 74.2 g (93.5%) of white crystals, m.p. 54-56°, and the recrystallization raised the melting point to 59-60°; infrared (KBr): 3320, 1725, 1695, 1535, and 1250 cm⁻¹.

\[ C_{15}H_{19}O_N \]

Calculated: C 69.03, H 7.62, N 11.07
Found: C 68.90, H 7.62, N 11.99

\[ N'-\text{(6-Carboxbenzoxaminocaprolyl)-p-N,N-dimethylphenylene-diamine (II)} \]—N,N'-Dicyclohexylcarbodiimide (5.75 g, 0.0279 mole) was added to a solution of N,N dimethylphenylenediamine (3.5 g, 0.0257 mole) and 7.0 g (0.0294 mole) of Compound I in 150 ml of acetonitrile (distilled over P2O5) and stirred at room temperature overnight. The white precipitate, dicyclohexylurea (4.4 g), was filtered away, and the filtrate was evaporated and replaced with boiling benzene (100 ml). It was treated with Norit, filtered, and the slightly colored crystalline product (9.8 g, 71.3%) was obtained upon cooling. Recrystallization from benzene and hexane gave white crystals, m.p. 113-114°; infrared (KBr): 3270, 1712, 1668, 1514, and 1232 cm⁻¹.

\[ C_{29}H_{37}O_N \]

Calculated: C 74.88, H 7.48, N 9.65
Found: C 74.84, H 7.44, N 9.73

\[ \text{(N-(6-Carboxbenzoxaminocaprolyl)-p-aminophenyl)trimethylammonium Iodide (III)} \]—Methyl iodide (10 ml, an excess) was added to a solution of Compound II (6.5 g, 0.017 mole) in 10 ml of dimethyl formamide and heated on a steam bath for 20 min. Ethyl acetate was added dropwise with swirling to the reaction flask until slight turbidity appeared. On cooling and rubbing the side of the flask with a glass rod, 8.5 g (96%) of white crystals, Compound III, m.p. 112-114°, were obtained. Infrared (KBr): 3270, 1712, 1668, 1514, and 1232 cm⁻¹.

\[ C_{34}H_{41}O_N \]

Calculated: C 74.88, H 7.48, N 9.65
Found: C 74.84, H 7.44, N 9.73

\[ \text{(N-(6-Amino-3-carboxbenzoxaminocaprolyl)-p-aminophenyl)trimethylammonium Bromide Hydrobromide (IV)} \]—Glacial acetic acid (13 ml) which had been saturated with gaseous anhydrous hydrogen bromide was added to 1 g of Compound III and stirred for 10 min. When there was no further sign of decarboxylation, 25 ml of anhydrous ethyl ether were added with swirling and allowed to stand for about 5 min to ensure the separation of gummy product. The supernatant was decanted, and the product was washed twice with 25 ml of ether. The resulting light brown, gummy material was crystallized from methanol and ethyl acetate to yield 0.78 g (97%) of white crystals, m.p. 205-207°, with decomposition. Treating the aqueous solution of the product with freshly prepared AgBr, filtering, evaporating, and following the recrystallization from methanol-ethyl acetate afforded an analytical sample, m.p. 209-210°; infrared (KBr): 3000, 1690, 1009, and 1540 cm⁻¹; ultraviolet: λmax 243 nm (ε, 14,900).

\[ C_{39}H_{45}O_N \]

Calculated: C 42.37, H 6.40, N 9.88, Br 37.58
Found: C 42.27, H 6.32, N 10.05, Br 37.56
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[N'- (6- Aminocaproyl)-p-N, N-dimethylphenylendiamine] dihydrobromide (V) — To 2.2 g (0.0057 mole) of Compound II were added 25 ml of saturated solution of dry hydrogen bromide in glacial acetic acid and stirred for about 10 min. Upon cessation of CO₂ evolution, 50 ml of anhydrous ether were added to filtered away, and the filtrate was evaporated. Sodium carbonate was added 25 ml of saturated solution of dry hydrogen bromide in acetone, and 3.71 g (0.018 mole) of dicyclohexylcarbodiimide were added to 100 ml of acetonitrile and stirred at room temperature overnight. The white precipitate, dicyclohexylurea, was filtered away, and the filtrate was evaporated. Sodium carbonate solution was added to the residue and extracted three times with chloroform (250 ml), dried with anhydrous sodium sulfate. Filtered and evaporated. The crude product was dissolved in boiling benzene, filtered (to remove a small amount of dicyclohexylurea), and crystallized on cooling with a small amount of hexane. Recrystallization from 50% benzene-hexane gave 3.87 g (59%) of white crystals, m.p. 106-109°C, infrared (KBr): 3300, 1700, 1670, 1530, and 1250 cm⁻¹.

C₁₄H₂₈N₄O₂
Calculated: C 47.04, H 7.19, N 10.36, Br 29.59
Found: C 46.97, H 6.86, N 10.87

[N'- (6-Carboxyaminoacaproyl)-6'-aminocaproyl]-p-N,N-dimethylphenylendiamine (VI) — Compound VIII was prepared in 89% yield from VII as described for Compound IV, white crystals, m.p. 128-130°C, obtained. Infrared (KBr): 3250, 1700, 1600, 1520, and 1260 cm⁻¹. Found: C 53.72, H 6.38, N 7.56

C₁₄H₂₈N₄O₂
Calculated: C 54.64, H 6.74, N 9.37
Found: C 54.04, H 6.74, N 9.37

[N'- (6-Carboxyaminoacaproyl)-6'-aminocaproyl)-p-amino- phenyl] trimethylammonium Bromide Hydrobromide (XIII) — Methyl iodide (0.5 ml) was added to a solution of Compound IX (0.265 g, 0.668 mmole) in 10 ml of absolute ethanol and heated under reflux on a steam bath for 20 min. Ethyl acetate was added dropwise until slight turbidity appeared. On cooling and rubbing the side of the flask with a glass rod, 0.355 g (98%) of white crystals, Compound X, m.p. 128-130°C, was obtained. Infrared (KBr): 3250, 1700, 1600, 1520, and 1250 cm⁻¹.

C₁₄H₂₈N₄O₂Br₂
Calculated: C 53.54, H 6.37, N 7.80
Found: C 53.72, H 6.38, N 7.56

Methyl- [N'- (6-Carboxyaminoacaproyl)-3-aminopyridine] Iodide (XIII) — Methyl iodide (0.5 ml) was added to 0.272 g (0.798 mmole) of pure Compound XII in 10 ml of ethanol and a few drops of dimethylformamide and heated under reflux for 30 min. Anhydrous ether was added dropwise until a slight turbidity appeared, and the flask was left in a refrigerator for 2 days with occasional rubbing of the side of the flask with a glass rod. The crystallization is slow and somewhat difficult, but finally it gave very pale crystals, Compound XIII, 0.347 g (90%). Recrystallization from ethanol and ethyl acetate gave m.p. 83-85°C. Infrared (KBr): 3200, 1690 (broad), 1538, and 1250 cm⁻¹.

C₁₄H₂₈N₄O₂Br₂
Calculated: C 46.35, H 7.11, N 10.40, Br 29.69
Found: C 46.85, H 7.11, N 10.36, Br 29.59

N'- (6-Carboxyaminoacaproyl)-p- amino N,N-dimethylben zylamine (IX) — The crude p-amino-α-dimethylaminotoluene (15), which was prepared from tin (5 g) and concentrated hydrochloric acid (12 ml) reduction on p-nitro-α-dimethylamino-
Methyl[\(N-(\beta-L{-aminocaproyl})-3{-amino}\)]pyridinium Bromide Hydrobromide (XIV)—Compound XIV was prepared from XIII as described for Compound IV, white crystals, m.p. 204°-205°; recrystallization after treating with AgBr gave an analytical sample, m.p. 205° (decomposed). Infrared (KBr): 2930, 1705, 1558 cm\(^{-1}\); ultraviolet: \(\lambda_{max} 262 \text{ nm (} \epsilon, 11,100)\) and 201 nm (\(\epsilon, 4,620)\).

\[
\text{C}_{20}\text{H}_{24}\text{O}_{2}\text{N}_{1}\text{Br}
\]

Calculated: C 49.70, H 5.42, N 8.69
Found: C 49.74, H 5.52, N 8.69

\[
\text{C}_{20}\text{H}_{24}\text{O}_{2}\text{N}_{1}\text{I}
\]

Calculated: C 49.70, H 5.42, N 8.69
Found: C 49.74, H 5.52, N 8.69

**Methods**

Coupling of Inhibitors to Sepharose—A modification of the procedure given by Cuatrecasas (12) was made in order to obtain a reproducible (10 to 15%) ligand concentration per ml of Sepharose and to conserve ligand. The following example is typical of the coupling procedure. A well washed suspension of 100 ml of Sepharose 4B on a Buchner funnel was transferred to a 200-ml beaker with 100 ml of distilled water and cooled to 4°. The ice bath was removed, a thermometer and a pH electrode were immersed in the beaker, and 1 g (9.45 mmoles) of CNBr was quickly transferred to a Buchner funnel and washed for 5 to 7 min under suction with 1 liter of cold 0.05 M carbonate-bicarbonate buffer (pH 9.8). The activated Sepharose was rapidly suspended in 80 ml of the above buffer solution containing 250 \(\mu\)moles of ligand and stirred overnight at 4°. Under these conditions the ligand is assumed to be in excess of the Sepharose activation sites. The substituted Sepharose was then washed with 1 liter of distilled water on a Buchner funnel and further washed extensively with distilled water until no ligand ultraviolet absorbance was detected in the washings. Table I summarizes the optimum ratios of materials used to obtain a prescribed level of ligand coupling for 25- to 250-ml quantities of Sepharose 4B.

**Table I**

**Coupling of ligand to Sepharose 4B**

The preparation of cyanogen bromide-activated Sepharose and conditions for the coupling of the inhibitor ligand, in this case either mono-(\(\beta-L{-aminocaproyl})\)-PTA or di-(\(\beta-L{-aminocaproyl})\)-PTA, are described under "Materials." The quantity of material required for the coupling procedure. A well washed suspension of 100 ml of Sepharose 4B on a Buchner funnel was transferred to a 200-ml beaker with 100 ml of distilled water and cooled to 4°. The ice bath was removed, a thermometer and a pH electrode were immersed in the beaker, and 1 g (9.45 mmoles) of CNBr was quickly transferred to a Buchner funnel and washed for 5 to 7 min under suction with 1 liter of cold 0.05 M carbonate-bicarbonate buffer (pH 9.8). The activated Sepharose was rapidly suspended in 80 ml of the above buffer solution containing 250 \(\mu\)moles of ligand and stirred overnight at 4°. Under these conditions the ligand is assumed to be in excess of the Sepharose activation sites. The substituted Sepharose was then washed with 1 liter of distilled water on a Buchner funnel and further washed extensively with distilled water until no ligand ultraviolet absorbance was detected in the washings.

<table>
<thead>
<tr>
<th>Added CNBr</th>
<th>Added ligand</th>
<th>Attached ligand</th>
<th>Coupling based on CNBr</th>
<th>Coupling based on ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/ml gel</td>
<td>(\mu)moles/ml gel</td>
<td>(\mu)moles/ml gel</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>0.7</td>
<td>1.5</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>2.5 to 3.0</td>
<td>2.0 ± 0.3</td>
<td>2.1</td>
<td>60 to 80</td>
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<tr>
<td>20</td>
<td>6.0 to 8.0</td>
<td>4.0 ± 0.6</td>
<td>2.1</td>
<td>60 to 80</td>
</tr>
</tbody>
</table>

**Benzylation DEAE-cellulose (B-DEAE-cellulose)—** The preparation of B-DEAE-cellulose and the washing procedures for this and other ion exchange celluloses have been described earlier (5). The solvent for the benzylation of DEAE-cellulose was modified to 200 ml of dimethylformamide and 800 ml of methyl cellosolve per 50 g of DEAE-cellulose. Microgranular DEAE-cellulose (Whatman) was not suitable for benzylation under these conditions because it is too soluble for adequate recovery.

**Crude Enzyme—** Lyophilized crude enzyme preparations were obtained in collaboration with Dr. A. L. Baker of Worthington. The crude enzyme had been extracted from toluene-treated tissue and prepared using ammonium sulfate precipitation as described in Steps 1 and 2 of Reference 6, a procedure which resulted in a 20 to 30 fold purification relative to enzyme in a fresh tissue homogenate. These crude preparations served as the starting material for all purification procedures described in this paper. They had essentially constant specific activities of 25 to 32.

**Enzyme Activity Assay—** Enzyme activities were measured by the pH-stat method with the use of a Radiometer titrator. The assay medium contained 0.1 M sodium chloride, 0.02 M magnesium chloride, 0.005% gelatin, and the substrate, 2.7 \(\mu\)mol recrystallized acetylcholine bromide (Eastman). The pH was adjusted to 7.4, and the temperature to 25°.

**Protein Determination—** Protein concentrations were routinely estimated spectrophotometrically, using either the absorbance at 280 nm or the absorbance difference between 215 and 225 nm. Extinction coefficients for purified acetylcholinesterase at these wave lengths were determined by relating the absorbance measurements to absolute protein values based on nitrogen determination, differential refractometry, and dry weight.

Nitrogen was determined by both the Dumas and the micro-Kjeldahl methods (Schwarzkopf Micronanalytical Laboratories, Woodside, N.Y.); nitrogen determinations on a micro-scale were also performed by means of a mnhhydrin complex-forming procedure (17). Nitrogen values were converted to protein with the use of the assumption that the protein contains 16.0% nitrogen.

The absolute protein contents of samples of protein carefully dialyzed against the solvent were determined by an interferometric technique, using a capillary type of synthetic boundary cell in the Spinco model E analytical ultracentrifuge as a differential refractometer. A value of 4.21 interference fringes per mg per ml was used and included a slight correction for the amino acid composition (18).

Dry weight measurements were carried out as described by Leuzinger et al. (8) on protein dialyzed for 3 days against 0.002 M ammonium carbonate. Several aliquots containing 50 to 300 \(\mu\)g of protein or appropriate blanks were dried at 80°, then repeatedly heated at 110° for 60 min and cooled in a desiccator over phosphorus pentoxide until the weights were constant.

An analysis of purified protein solutions for the presence of sugar was carried out (10) with sucrose as a standard at two different phenol concentrations. Data were collected in terms of the phenol concentration which gave the higher percentage of sugar estimate, and a slight protein blank correction was made from control analyses of highly purified chymotrypsin (Worthington), lysozyme (Worthington), and bovine serum albumin (Sigma).

**Purification by Affinity Chromatography—** Stock solutions of 0.02 M potassium phosphate buffer at pH 6.9 and 0.5 M NaCl in
buffer were prepared and filtered with a Millipore apparatus. From this point all steps were carried out at 3–5°C. A solution of 3.6 to 5.0 g of lyophilized crude enzyme dissolved in 80 ml of stock buffer was dialyzed against 10 liters of stock buffer for 24 hours at 5°C. A slight turbidity was removed from the enzyme solution by centrifugation at 18,000 × g for 20 min. Aliquots of this crude enzyme stock solution were then added to a chromatographic column of the substituted Sepharose which had been washed and packed in stock buffer. The crude enzyme aliquot was allowed to equilibrate with the column overnight. The aliquot addition was usually followed by further washing with stock buffer until the nonretained material had been completely eluted and the 280 nm absorbance of the washings had dropped to zero. Elution of the retained enzyme was accomplished by a buffered linear gradient of sodium chloride or potassium chloride, starting either from zero salt concentration or from a low salt concentration which had been used to further wash weakly retained impurities from the column.

The column eluent was continuously monitored at 254 nm with an LKB Uvicord I. Fractions were assayed both for enzyme activity on the pH-stat and for protein spectrophotometrically. Active fractions were pooled and concentrated by a vacuum dialysis procedure which uses a collodion membrane bag (Schleicher and Schuell, Inc.) for subsequent characterization studies.

**Purification by Standard Chromatographic Procedures**—These studies were made generally as outlined in the detailed chromatographic methods given in Steps 2 through 6 of the procedure of Kremzner and Wilson (5). However, the following modifications of these procedures were applied in our work. A concentration of 0.03 M sodium phosphate buffer was used for equilibration and elution of all columns. All steps were carried out at 3–5°C. The lyophilized crude enzyme was dissolved in 2.5% ammonium sulfate (2 to 10 mg of protein per ml); insoluble material was removed by centrifugation at 25,000 × g for 10 min; and the mixture was applied to a B-DEAE-cellulose column (3 to 12 mg of protein per ml of packed resin) in Step 2. A solvent higher in ionic strength than that in the previous procedure was used to minimize the presence of soluble protein aggregates which may interfere with purification on B-DEAE-cellulose. The pooled enzyme, eluted with a stepwise sodium chloride gradient in Step 2, was concentrated (Step 3) by the LKB Uvicord I. Fractions were assayed both for enzyme activity and for protein on a Beckman amino acid analyzer.

Equilibrium dialyses of 0.5–1.0-ml enzyme samples (0.5 to 1.0 mg per ml) in 9-mm dialysis tubing were carried out for 20 hours at 1–5°C with shaking in 75 ml of solvent containing 2 to 200 μl of either [14C]Bis-Q or a mixture of [14C]Bis-Q and Bis-Q. The solvent for the titration was either 0.1 M NaCl in pH 6.85 buffer (0.05 M phosphate) or normal eel Ringer’s minus glucose (21) at pH 7.0. Aliquots of 100 to 500 μl were taken from both the inside and the outside of the equilibrated dialysis solutions and added to 10-ml quantities of a slightly modified Bray’s scintillation cocktail (22) for counting on a Packard model 574 scintillation counter. Further additions to the counting vials were made in some cases to make the total composition of added sample in these vials identical, except for the quantity of [14C]Bis-Q, but such precautions to insure reproducible counting efficiencies had little effect on the results. A weighted least squares computer analysis of reciprocal plot data was used to quantitatively determine the binding parameters, and these values were in agreement with those estimated from Scatchard plots.

**Immunoelectrophoresis**—This procedure was carried out in a 1.5% agar matrix (23, 24) with the use of sodium barbitol buffer (0.05 M, pH 8.3). The voltage was adjusted to 20 volts per cm, and the sample was run for 100 min.

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis patterns were obtained in glass tubes (6 × 150 mm) in a Buchler apparatus thermostatted at 10°C. A procedure based on the alternate method of Davis (25) was followed, using Tris-glycine buffer at pH 8.3, 7% polyacrylamide in the separation gel, and 1.25% polyacrylamide in the 0.5-cm spacer gel (reagents from Canalo and Eastman). Samples were added to a top 20-μl layer of 40% sucrose which contained bromphenol blue as a tracking dye.

Gels were protein-stained for 20 min with Amido Schwarz; staining for esterase activity was carried out in phosphate buffer (0.05 M, pH 7.0) and was based on the diazo blue B reaction with β-naphthol released during hydrolysis of β-naphthyl acetate (24). Gels were destained by diffusion in 7% acetic acid. Relative band intensities were measured on a Canalo model E microdensitometer.

**Error Analysis**—The analysis of variance in weighted least squares plots was carried out as outlined previously (13). The standard error (S.E.) is listed with several tabulated values. This quantity is the square root of the variance of the mean
value and is given by the expression
\[
S.E. = \sqrt{\frac{\sum w_i(z_i - \bar{z})^2}{\sum w_i(n - 1)}}
\]
where \(z_i\) is an individual observation; \(\bar{z}\) is the mean of the \(z_i\) values; \(w_i\) is the weight given to an individual observation; and \(n\) is the number of observations. A weighted analysis was used

### TABLE II

Competitive inhibition dissociation constants (\(K_I\)) for several inhibitors of acetylcholinesterase

| Inhibitor                     | \(K_I\)  
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Mono-(6-aminocaproyl)-PTA</td>
<td>39 ± 9</td>
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<tr>
<td>Mono-(6-aminocaproyl)-PTA-Sepharose</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>Di-(6-aminocaproyl)-PTA</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>Di-(6-aminocaproyl)-PTA-Sepharoseb</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Mono-(6-aminocaproyl)-BTA</td>
<td>52 ± 37</td>
</tr>
<tr>
<td>Mono-(6-aminocaproyl)-MP</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>Bis-Q</td>
<td>4.6 ± 0.2</td>
</tr>
</tbody>
</table>

*\(K_I\) values estimated from weighted least squares plots (13) (see also under "Methods").

b. Attainment of steady-state inhibition was slow with these gel-linked inhibitors. Enzyme was pre-equilibrated with the final concentration of inhibitor gel for 30 to 45 min prior to each rate measurement.

Inhibition of Enzyme Activity—The competitive inhibition of acetylcholinesterase activity by the ligands synthesized for use in affinity chromatography is shown in Table II. The characteristic inhibition patterns of the ligand are altered only slightly on attachment to the gel; no significant differences are observed among the various PTA derivatives except for the di-(6-aminocaproyl)-PTA-Sepharose, which apparently binds enzyme 2 to 3 times more tightly than the free ligand. The free mono-(6-aminocaproyl)-MP ligand inhibits the enzyme with a competitive \(K_I\) which is about 10 times lower than the PTA derivatives and therefore may be more selective in affinity chromatography. Studies using this inhibitor with extension arms of various lengths are currently in progress.

Affinity Chromatography—A preliminary affinity chromatography purification using the di-(6-aminocaproyl)-PTA-Sepharose is shown in Fig. 1. The enzyme is the last detectable protein component to be eluted from the column, and it is released to eluting buffer containing about 0.2 M NaCl. While the resolution of enzyme from protein contaminants appearing earlier in the elution profile may be improved by an increased period of washing with stock buffer, the preliminary chromatographic run in Fig. 1 demonstrates a sharp rise in specific activity of eluted enzyme to a constant value corresponding to that of essentially homogeneous enzyme (see below).
The per cent yield is expressed relative to the applied crude enzyme solution, which had a specific activity of 25 to 32 and an assay pH of March 10, 1972. L. Rosenberry, H. W. Chang, and Y. T. Chen. The per cent yield is expressed relative to the applied crude enzyme solution, which had a specific activity of 25 to 32 and an assay pH of March 10, 1972. L. Rosenberry, H. W. Chang, and Y. T. Chen.

Di-(6-aminocaproyl)-PTA

**Table III**

**Summary of affinity chromatography purification**

The chromatographic procedure is described under "Methods." The per cent yield is expressed relative to the applied crude enzyme solution, which had a specific activity of 25 to 32 and an assay pH of March 10, 1972. L. Rosenberry, H. W. Chang, and Y. T. Chen.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-(6-aminocaproyl)-PTA</td>
<td>520-575</td>
<td>35-75</td>
</tr>
<tr>
<td>Di-(6-aminocaproyl)-PTA</td>
<td>560-610</td>
<td>65-75</td>
</tr>
<tr>
<td>Mono-(6-aminocaproyl)-PTA</td>
<td>380-420</td>
<td>40-60</td>
</tr>
</tbody>
</table>

* The quantity of crude enzyme added (6.7 mmoles per min per ml of ligand) was that observed to be just sufficient to saturate the Sepharose, which contained 1.61 μmoles of di-(6-aminocaproyl)-PTA per ml of packed gel. Under these conditions the nonretained enzyme usually did not exceed 15% of the total applied enzyme. The wide range in the yield reflects recoveries from both linear and nonlinear salt gradient eluitions. Nonlinear salt gradients gave generally better resolutions, and recoveries from these column runs were always in excess of 60%.

**Table IV**

**Summary of standard chromatography purification procedures**

The chromatographic procedure is described under "Methods." The per cent yield is expressed relative to the applied crude enzyme solution, which had a specific activity of 25 to 32 and an assay pH of March 10, 1972. L. Rosenberry, H. W. Chang, and Y. T. Chen.

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Specific activity</th>
<th>Yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Extraction and ammonium sulfate precipitation</td>
<td>31 (30-32)</td>
<td>100 (defined)</td>
</tr>
<tr>
<td>2</td>
<td>B-DEAE-cellulose</td>
<td>140 (100-225)</td>
<td>52 (44-62)</td>
</tr>
<tr>
<td>3</td>
<td>Concentration and dialysis</td>
<td>145</td>
<td>50 (48-51)</td>
</tr>
<tr>
<td>4</td>
<td>Sephadex G 200</td>
<td>350 (190-310)</td>
<td>43 (11-16)</td>
</tr>
<tr>
<td>5</td>
<td>Cellulose-P</td>
<td>410 (340-470)</td>
<td>25 (23-26)</td>
</tr>
<tr>
<td>6</td>
<td>DEAE-cellulose</td>
<td>475 (460-490)</td>
<td>16 (15-18)</td>
</tr>
<tr>
<td>7</td>
<td>DEAE-Sephadex</td>
<td>500 (480-510)</td>
<td>12 (12-13)</td>
</tr>
<tr>
<td>8</td>
<td>DEAE-cellulose-I</td>
<td>520 (510-540)</td>
<td>9 (8-10)</td>
</tr>
</tbody>
</table>

* The first number is the average over-all specific activity for the purification step, and the numbers in parentheses are the average range of specific activities pooled.

**Fig. 2 again uses the di-(6-aminocaproyl)-PTA gel, but here the column was run at pH 7 instead of 5. The chromatography on B-DEAE-cellulose (Step 2) revealed that a major component of the crude enzyme preparation was retained with greater affinity than acetylcholinesterase. Although at low levels of column loading (1.2 mmoles per min per ml of packed resin) purification was not hindered, higher loading levels decreased both the purification and the yield. At high loading levels (5.0 mmoles per min per ml of packed resin), this contaminant displaced up to 75% of the applied enzyme prior to the initiation of the salt gradient. The eluted enzyme could be rechromatographed on B-DEAE-cellulose.
FIG. 3 (top left). Polyacrylamide gel electrophoresis of crude and purified preparations of acetylcholinesterase (E). The enzyme had been purified by affinity chromatography on di-(6-aminocaproyl)-PTA-Sepharose to a specific activity of 490. Gels were run at 7 volts per cm until the tracking dye reached the bottom of the gel, or about 8 hours. The applied samples, reading from the left, were (1) 8.6 µg of purified E stained for esterase activity; (2) 8.6 µg of purified E stained for protein; (3) 86 µg of purified E stained for protein; (4) 50 µg of crude E stained for esterase activity; (5) 50 µg of crude E stained for protein.

FIG. 4. (bottom left). Polyacrylamide gel electrophoresis of purified acetylcholinesterase at increasing sample loads. The enzyme had been purified as in Fig. 3 to a specific activity of 520. Gels were run at 2.5 mA per tube for 8 hours; the spacer gel was omitted. The applied samples, reading from the left, were (1) 8.6 µg; (2) 43 µg; (3) 86 µg.

FIG. 5 (right). Immunoelectrophoresis of acetylcholinesterase at various stages of purification. Antisera both to a crude enzyme extract (specific activity 2 to 3) and to partially purified enzyme (specific activity 192) which had been prepared previously (27) were applied to 40- to 100-µg protein samples which had been subjected to electrophoresis as described under “Methods.” a, upper trough, antisera to the crude extract (Anti-C); well, enzyme purified on di-(6-aminocaproyl)-PTA-Sepharose to a specific activity of 520; lower trough, antisera to the partially purified enzyme (Anti-P). b, upper well, enzyme partially purified by standard chromatographic procedures with a specific activity of 340; trough, Anti-P; lower well, enzyme partially purified by standard chromatographic procedures with a specific activity of 330. (Enzyme in b was obtained from a fractionation on DEAE-Sephadex and was prepared by Dr. G. Lovinger.) c, upper trough, anti-C; upper well, crude enzyme extract of specific activity 2 to 3; lower trough, mixture of equal parts of Anti-C and Anti-P; lower well, enzyme purified first by standard chromatographic procedures and further run on mono-(6-aminocaproyl)-PTA-Sepharose with a specific activity of 550.

The elution volume corresponding to the peak acetylcholinesterase activity during chromatography of the partially purified enzyme on Sephadex G-200 was used to estimate the molecular weight of the enzyme (26). The observed value, based on three chromatographic runs, was 245,000 ± 10,000.

**Polyacrylamide Gel Electrophoresis**—The homogeneity of enzyme purified on di-(6-aminocaproyl)-PTA-Sepharose (Table III) was determined both by polyacrylamide gel electrophoresis and by immunoelectrophoresis procedures. In Fig. 3 the gel electrophoresis patterns of enzyme purified to a specific activity of 490 are compared with those of the crude enzyme applied to affinity chromatography. This enzyme had lost activity on storage from an original specific activity of 520 to the value of 490 obtained on the day of the electrophoresis run. No bands other than an active esterase band are observed when an 8.6-µg protein sample is run; however, at the higher 86-µg protein load a faint diffuse band of inactive impurity becomes apparent which corresponds to 10% of the total applied protein, according to a microdensitometer trace analysis. Essentially all the protein in the purified sample passes through the space gel and enters the stacking gel. Gel electrophoresis patterns of purified enzyme with a higher specific activity of 520 in Fig. 4 demonstrate that an impurity, here corresponding to 10% of the total applied protein, may only be observed with high protein load.
Table V

Extinction coefficients based on several methods of absolute protein determination

The protein content of various samples of purified acetylcholinesterase having specific activity of 520 to 560 was determined by the following techniques, described in detail under "Methods." With the use of the corresponding absorbance at 280 nm of the sample, the extinction coefficient was obtained. No differences in extinction coefficient were found among samples whose specific activity varied from 520 to 560 by any of the techniques.

<table>
<thead>
<tr>
<th>Protein determination</th>
<th>Number of determinations</th>
<th>( \epsilon \text{ cm}^{-1} \text{ mg}^{-1} \text{ ml} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Analysis of nitrogen content by the Dumas or micro-Kjeldahl method (^a)</td>
<td>4</td>
<td>21.4 ± 0.3</td>
</tr>
<tr>
<td>2. Analysis of nitrogen content by ninhydrin micro-method (^b)</td>
<td>4</td>
<td>21.8 ± 0.7</td>
</tr>
<tr>
<td>3. Analysis of nitrogen content from the amino acid composition (^c)</td>
<td>2</td>
<td>18.8 ± 0.5</td>
</tr>
<tr>
<td>4. Differential refractometry</td>
<td>2</td>
<td>17.6 ± 0.3</td>
</tr>
<tr>
<td>5. Dry weight</td>
<td>6</td>
<td>18.2 ± 1.1</td>
</tr>
</tbody>
</table>

\(^a\) The extinction coefficient at 280 nm is defined as the absorbance of a solution containing 10 mg of protein per ml and is listed as the mean and standard error for the number of determinations shown. The difference absorbance (\( \Delta \epsilon_{280} \text{ cm}^{-1} \text{ mg}^{-1} \text{ ml} \)) may also be used to estimate the protein concentration. The ratio of this difference extinction coefficient to \( \epsilon_{280} \text{ cm}^{-1} \text{ mg}^{-1} \text{ ml} \) was very reproducible for samples highly purified either by affinity or by standard chromatographic procedures and was observed to be 3.52 ± 0.05.

\(^b\) The corresponding ratio (\( \frac{\Delta \epsilon_{280} \text{ cm}^{-1} \text{ mg}^{-1} \text{ ml}}{\epsilon_{280} \text{ cm}^{-1} \text{ mg}^{-1} \text{ ml}} \)) was 2.1 ± 0.1.

\(^c\) Conversion of nitrogen to protein was made assuming that the protein contained 10.60% nitrogen.

Immunoelectrophoresis—Patterns obtained at various stages of enzyme purification are shown in Fig. 5. While antisera to crude enzyme measuredly precipitated only protein impurities, antisera to partially purified enzyme precipitated both enzyme and certain impurities remaining at relatively high levels of purification. Only a single band is observed for enzyme purified to specific activities of 520 and 550, consistent with essentially homogeneous enzyme.

Protein Determination—A careful determination of the specific activity of the homogeneous protein demonstrated above required that the spectrophotometric protein determination be directly related to an absolute protein analysis. Three different techniques for measuring absolute protein content were employed, and the corresponding spectrophotometric extinction coefficients obtained from each technique are listed in Table V. A significant difference in protein estimates is obtained between techniques measuring micro quantities of nitrogen as \( \text{NH}_3 \) or \( \text{N}_2 \) and those monitoring dry weight or refractive index. The nitrogen analysis from the amino acid composition data (Table VI) tends to support the lower extinction coefficient obtained from the dry weight and refractive index. It appears that micro determinations of \( \text{NH}_3 \) or \( \text{N}_2 \) are underestimating the protein content by 10 to 15%. The difference could be real if the protein contained significantly less than the 16.0% nitrogen assumed if contaminants containing little nitrogen were present. (An analysis of the purified protein sample for polysaccharides and their derivatives with free or potentially free reducing groups (19) did give a positive reaction equivalent to about 4 to 5% sugar. This could arise from carbohydrate contamination from the Sepharose itself.) However, the fact that the nitrogen analysis from the amino acid composition data is in reasonable agreement with the protein estimated from the dry weight or the refractive index does not support this explanation and seems to indicate that relatively less importance should be attached to the micro determinations of nitrogen as estimates of protein content. A value of 18.0 ± 0.4 was assumed for the extinction coefficient at 280 nm.

Amino Acid Composition—Table VI shows the amino acid composition analysis obtained for enzyme purified on di-(\( \beta \))-aminocaproyl)-PTA-Sepharose. The average number of residues for each amino acid may be compared with values reported previously (6). Agreement within 1 residue is observed in all cases except proline, where the current analyses show 4 fewer residues per an arbitrary 20,100 molecular weight unit.

Active Site Titrations—The normality of enzyme catalytic sites was determined both by direct spectrophotometric titra-

---

Table VI

Amino acid composition of acetylcholinesterase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per 12 valine residues(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample</td>
</tr>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.1</td>
</tr>
<tr>
<td>Ammonia</td>
<td>37.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.1</td>
</tr>
<tr>
<td>Half-cystine (as cysteic acid)</td>
<td>3.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>21.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.3</td>
</tr>
<tr>
<td>Serine</td>
<td>11.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>17.6</td>
</tr>
<tr>
<td>Proline</td>
<td>8.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>15.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.2</td>
</tr>
<tr>
<td>Valine</td>
<td>12</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>13.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.6</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.4</td>
</tr>
</tbody>
</table>

\(^d\) Data are tabulated in terms of valine, which showed a constant mole per cent based on total amino acid content of 7.05 ± 0.08. Twelve valine residues were used as a reference number to facilitate comparison with a previous report (6). The total amino acid residues reported thus correspond to a polypeptide of molecular weight 20,100. Data appear as observed values uncorrected for any decomposition occurring during the 24-hour hydrolysis.

\(^*\) The sealed hydrolysis tube contained oxygen allowing a cysteic acid determination.

\(^*\) Samples subjected to performic acid oxidation according to the method of Hirs (28).

\(^*\) Estimated spectrophotometrically (29).
The titration conditions are described under "Methods," and the calculations are explained under "Results." In each case enzyme was purified on di-(6-aminocaproyl)-PTA-Sepharose.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Turnover number</th>
<th>Apparent sites per molecule</th>
<th>Corrected sites per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>M7C*</td>
<td>7.66 ± 0.25 x 10*</td>
<td>2.85 ± 0.13</td>
<td>3.44 ± 0.21</td>
</tr>
<tr>
<td>M7C'</td>
<td>8.03 ± 0.26 x 10*</td>
<td>3.15 ± 0.15</td>
<td>3.28 ± 0.20</td>
</tr>
<tr>
<td>Bis-Q*</td>
<td>7.63 ± 0.59 x 10*</td>
<td>2.92 ± 0.24</td>
<td>3.46 ± 0.31</td>
</tr>
<tr>
<td>Bis-Q'</td>
<td>8.00 ± 0.32 x 10*</td>
<td>2.81 ± 0.21</td>
<td>2.98 ± 0.25</td>
</tr>
</tbody>
</table>

* Assumes the protein solution is 100% active acetylcholinesterase and that the enzyme molecular weight is 260,000 ± 10,000.

** Corrects for contaminant or inactive protein by normalizing the apparent value at the observed specific activity to the value corresponding to a pure enzyme solution with a specific activity of 610 ± 15.

** Enzyme specific activity was 555 ± 15; the protein concentration was 14.4 ± 0.19 μg per ml; and the observed normality was 1.58 ± 0.03 x 10-7 N.

** Enzyme specific activity was 555 ± 10; the protein concentration was 25.9 ± 0.55 μg per ml; and the observed normality was 3.14 ± 0.05 x 10-7 N.

** Enzyme specific activity was 515 ± 15; the protein concentration was 201 ± 27 μg per ml; and the observed normality was 163.1 ± 6.9 x 10-7 N.

** Enzyme specific activity was 575 ± 15; the protein concentration was 180 ± 19 μg per ml; and the observed normality was 102.6 ± 6.1 x 10-7 N.

An attractive feature of the use of M7C as a titrating agent is equilibrium dialysis with Bis-Q as described under "Methods." From the normality one may calculate both the turnover number for enzyme-catalyzed acetylcholine hydrolysis and, if the molarity of the enzyme solution can be established, the number of enzyme catalytic sites per molecule. To determine the enzyme molarity one must know both the mg of active enzyme per ml and the enzyme molecular weight. In this study the molecular weight was assumed to be 260,000, consistent with the gel filtration value reported above and with values measured by sedimentation equilibrium (30, 31). The total protein concentration was determined spectrophotometrically; and the active enzyme concentration was calculated from the specific activity of the solution, assuming pure enzyme to have a specific activity of 610. This sequence of calculations is shown in Table VII and leads to an average value of 3.29 ± 0.11 active sites per molecule of enzyme.

An attractive feature of the use of M7C as a titrating agent is the fact that the entire course of the carbamoylation reaction may be observed directly. Hence the following kinetic parameters, rigorously defined in a previous paper (13), were observed for the titration reaction: the specific carbamoylation rate constant, k₅, was 5.1 ± 0.8 min⁻¹; the specific decarbamoylation rate constant, k₉, was 0.033 ± 0.010 min⁻¹; and the pre-carbamoylation (reversible) titrant dissociation constant, Kₑ, was 6.7 ± 1.8 μM. These values are identical, within experimental error, with those reported previously for enzyme of specific activity 70 (13) and thus support the conclusion that the specific catalytic rate constants for acetylcholinesterase are not altered by affinity chromatography.

A typical result from the equilibrium titration of acetylcholinesterase with Bis-Q is shown in Fig. 6. The average dissociation constant, Kᵦ, observed for the complex of Bis-Q with enzyme was 5.4 ± 0.5 μM, and complex formation was completely reversible. This Kᵦ agrees with the competitive inhibition constant K_I reported in Table V for the complex of Bis-Q with enzyme.

** Corrects for contaminant or inactive protein by normalizing the apparent value at the observed specific activity to the value corresponding to a pure enzyme solution with a specific activity of 610 ± 15.

A specific activity of 660 for acetylcholinesterase highly purified by the standard chromatographic procedures (Table IV) was observed by Kremmer and Wilson (5). However, relative ultraviolet absorption values given for this preparation (ε₃₄₃₅ = 22.9; Δ (ε₃₄₃₅ - ε₃₄₃₅)/ε₃₄₃₅ = 2.78; ε₃₄₃₅/ε₃₄₃₅ = 1.67 (7)) do not agree with the values found here (Table V). If the assay conditions and extinction coefficient assumed here are used to recalculate the reported specific activity of 660, a value of 550 is obtained, in good agreement with the maximum value of 540 observed for the standard purification procedures here, but slightly less than that obtained with affinity chromatography. Leuzinger and Baker (6) report a specific activity of 730 for enzyme purified by the standard chromatographic procedures; but a more recent report by Leuzinger (32) finds the same specific activity by means of a higher assay pH and a higher protein extinction coefficient, conditions under which one would expect the specific activity to differ by 25 to 30%.

The difficulties inherent to obtaining the specific activity of purified homogeneous enzyme may not be trivial, apart from the
fact that there may be experimental bias toward reporting higher specific activities. Highly purified enzyme has been reported to be somewhat unstable (5), and we have observed that the stability of enzyme above a specific activity of 500 varies greatly from preparation to preparation. In general, the stability is best preserved in a frozen solution of high enzyme concentration. A second inherent difficulty, which was illustrated under “Results,” is the method of protein determination used (Table V).

These difficulties are reflected in attempts to determine the stoichiometry of enzyme-active sites. A simultaneous determination of the turnover number per site is highly recommended, because this number is independent both of the protein concentration and the enzyme specific activity. Turnover numbers reported in Table VII agree with a series of turnover numbers for acetylcholinesterase obtained with a wide variety of titrants tabulated previously (13), a condition which we feel should be met for all active site stoichiometry determinations. Furthermore, it was shown previously that if a site turnover number, an enzyme molecular weight, and a specific activity for pure enzyme are assumed, it necessarily follows that a unique solution for the number of active sites per molecule may be found (13). This solution for the data in Table VII is 3.3 ± 0.1 active sites per molecule. Similar logic applied to a recent report of two active sites per molecule (32) would require a site turnover number about twice that indicated in Table VII, in disagreement with all previous turnover number determinations. Previous reports of four active sites per molecule (7, 13) were based on higher reported specific activities for pure enzyme of 660 and 730, values which have not been confirmed in this study.

A value of three active sites per molecule is somewhat disconcerting in view of the symmetry considerations that seem to dictate an even number of active sites for oligomeric enzymes (33). The most uncertain data entering into this calculation is the specific activity of pure enzyme. If specific activities higher than 610 can be demonstrated, the number of active sites per molecule would increase. Since we have not been able to demonstrate such higher activities, the possibility of three active sites per molecule must be seriously considered. In this regard, recent reports of subunit molecular weights corresponding to six subunits per molecule (31), rather than the four previously reported (30), are of interest, because three active sites per hexamer would indicate higher symmetry than three active sites per tetramer.

Acknowledgments—We express sincere appreciation to Professor David Nachmansohn for his advice and encouragement during the course of this study. We also gratefully acknowledge the considerable contributions made by Dr. Vacek Haris-dangkul to the immuno-electrophoresis and nitrogen determination results and by Dr. William Poillon to the differential refractometry measurement.

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Purification of Acetylcholinesterase by Affinity Chromatography and Determination of Active Site Stoichiometry
Terrone L. Rosenberry, Hai Won Chang and Yueh T. Chen


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