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 $\epsilon^\text{max}_{180}$ = 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 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 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 chromographic 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 (Cellex-P) was purchased from Bio-Rad, Richmond, California; and Sephadex and Sepharose gels came from Pharmacia.

Titrating Agents—The synthesis of N-methyl-(7-dimethylcarbamoyl)-quinolinium iodide has been described earlier (13). 3,3'-bis[N-(trimethylammonium)methyl]-azobenzene iodide, whose synthesis has been described (14), and 3,3'-bis[N-(trichloroethyl)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 in the trans-isomer predominates to greater than 90% (14), \( \lambda_{\text{max}} \) 316 nm (\( \epsilon \), 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°. Carboxbenzoxylchloride (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 carboxbenzoxylchloride, 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 NaSO4. 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\(^{-1}\).

**Calculation**

\[ C_{11}H_{19}O_{2} \]

Calculated: C 69.03, H 7.62, N 11.07

Found: C 69.03, H 7.62, N 11.07

\( [N-(6-Carboxbenzoxaminocaproyl) \cdot p\text{-aminophenyl}]\text{trimethylammonium iodide (III)} \)—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, 1514, and 1232 cm\(^{-1}\).

\[ C_{22}H_{33}N_{2}O_{3} \]

Calculated: C 71.37, H 7.01, N 16.10

Found: C 71.37, H 7.01, N 16.10

\([N-(6-Aminocaproyl) \cdot p\text{-aminophenyl}]\text{trimethylammonium bromide hydrobromide (IV)} \)—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.

**Titrating Agents**

The following abbreviations are used: mono-(6-aminocaproyl)-PTA, [N-(6-aminocaproyl)p-aminophenyl]trimethylammonium bromide hydrobromide (V); di-(6-aminocaproyl)-PTA, [N-(6-aminocaproyl)-6'-aminocaproyl]p-aminophenyl]trimethylammonium bromide hydrobromide (VIII); Bis-Q, 3,3'-bis[N-(trimethylammonium)methyl]-azobenzene bromide; mono-(6-aminocaproyl)-MP, methyl-[N-(6-aminocaproyl)-3-aminoipyridinium]trimethylammonium bromide; M7C, N-methyl-[N-(7-dimethylcarbamoyl)-quinolinium iodide; [\( ^{14} \text{C} \)] Bis-Q, 3,3'-bis[\( ^{14} \text{C} \)] trimethylammonium]-methyl]-azobenzene iodide; mono-(6-aminocaproyl)-BTA, [N-(6-aminocaproyl)p-aminophenyl]trimethylammonium bromide hydrobromide (XI).
[\textit{N'-(6-Aminocaproyl)-p,N,N-dimethylphenylenediamine}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 CO$_2$ evolution, 50 ml of anhydrous ether were added to filtered away, and the filtrate was evaporated. Sodium carbonate and glacial acetic acid were added, and the mixture was stirred for about 10 min. Upon cessation overnight. The white precipitate, dicyclohexylurea, was added 25 ml of saturated solution of dry hydrogen bromide in 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. Re crystallization from 50\% benzene-hexane gave 3.87 g (59\%) of white crystals, m.p. 105–109$^\circ$; infrared (KBr): 3300, 1700, 1670, 1530, and 1250 cm$^{-1}$.

\[ C_{14}H_{29}N_3O_2Br_2 \]
Calculated: C 43.92, H 6.64, Br 36.39
Found: C 43.92, H 6.64, Br 36.39

\[ N'-(6-Carbobenzoxyaminocaproyl)-p-amino-N,N-dimethylbenzylamine (IX) \] —The crude p-amino-\(\alpha\)-dimethylaminomandelic acid (15) was prepared from a mixture (5 g) and concentrated hydrochloric acid (12 ml) reduction on p-nitro-\(\alpha\)-dimethylaminotoluene (16) (3 g, 0.0168 mole), 4.4 g (0.0168 mole) of Compound I, 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. 105–109$^\circ$; infrared (KBr): 3300, 1700, 1670, 1530, and 1250 cm$^{-1}$.

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**METHODS**

**Coupling of Inhibitors to Sepharose**—A modification of the procedure given by Cuatrecasas (12) was made in order to attain 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°C. 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 added at once. The pH of the solution was quickly adjusted to about 7 to 8 min, and the final temperature of the reaction mixture was about 15°C. Ice was added to bring the temperature down, and the mixture 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 buffer (pH 9.8). The activated Sepharose was rapidly suspended in 80 ml of the above buffer solution containing 250 µmoles of ligand and stirred overnight at 4°C. 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**

<table>
<thead>
<tr>
<th>Added CNBr (mg/ml gel)</th>
<th>Added ligand (µmoles/ml gel)</th>
<th>Attached ligand (µmoles/ml gel)</th>
<th>Coupling based on CNBr (%)</th>
<th>Coupling based on ligand (%)</th>
</tr>
</thead>
<tbody>
<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 ± 30</td>
</tr>
<tr>
<td>20</td>
<td>6.0 to 8.0</td>
<td>4.0 ± 0.6</td>
<td>2.1</td>
<td>60 ± 30</td>
</tr>
</tbody>
</table>

**Benzylated 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 benzylated 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 carried through an ammonium sulfate precipitation as described in Steps 1 and 2 of Reference 6, a procedure which results 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 mM recrystallized acetylcholine bromide (Eastman). The pH was adjusted to 7.4, and the temperature to 25°C.

**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 mnhydrin 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 Spineo 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 µg of protein or appropriate blanks were dried at 80°C, then repeatedly heated at 110°C 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 (19) 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 albumen (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 vacuum dialysis procedure described above. This sample was run through a Sephadex C-200 column (1 ml of applied sample per 50 ml of packed gel) in Step 4.

The pH of the enzyme solution and of the column equilibration was adjusted from the usual 7.0 to 5.7 for cation exchange chromatography on Cellex-P (Step 5) immediately prior to application of the sample (0.2 to 0.3 mg of protein per ml of packed resin). Enzyme was eluted with a stepwise sodium chloride gradient, immediately readjusted to pH 7.0, and concentrated by vacuum dialysis.

The final purification steps were carried out with a combination of anion exchange columns which could be used interchangeably. The resins used in these steps were DEAE-cellulose, DEAE-Sephadex, and QAE-Sephadex. The applied sample protein per column volume was the same as that used in Steps 6 and 7 of Reference 5.

**Amino Acid Composition**—Separate analyses were performed on three 0.5-mg samples of purified and concentrated enzyme as described by Moore and Stein (20). To each dried sample was added 0.5 ml of fresh 6 N HCl, and the hydrolyses were carried out at 110°C for 24 hours. The analyses were performed on a Beckman amino acid analyzer.

**Competitive Inhibition Constants**—These were determined by computer analysis of reciprocal plot data (13) in which both the acetylcholine substrate and the inhibitor concentrations were varied.

**Active Site Titration**—Enzyme-active site normalities were determined either by fluorescent titration with the carbamoylating agent M7C8 as described in Reference 13 or by equilibrium dialyses using [3H]Bis-Q. The fluorescent titrations were measured at 25°C in pH 6.85 buffer (0.05 M phosphate) containing 0.1 M NaCl and were monitored on a Tracer-Bowman spectrophotofluorometer with dual monochromators. Kinetic parameters for the carbamoylation reaction were also determined as in the previous report (13).

Equilibrium dialyses of 0.5- to 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 μg of either [3H]Bis-Q or a mixture of [3H]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 ed 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 571 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 [3H]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 ordinary 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 (8 × 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 Canalco and Eastman). Samples were added to a 10-μl layer of 40% sucrose which contained bromphenol blue as a tracking dye.

Gels were protein-stained for 20 min with Anido schwars; 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 Canalco 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 only when each weight could be determined experimentally as the reciprocal of the variance of the given observation. In unweighted averaging, \( w_i = 1 \) for all \( i \).

### RESULTS

#### 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

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\(^1\) of eluted enzyme to a constant value corresponding to that of essentially homogeneous enzyme (see below).

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**Table II**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( K_I ) μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono-(6-aminocaproyl)-PTA</td>
<td>39 ± 9</td>
</tr>
<tr>
<td>Mono-(6-aminocaproyl)-PTA-Sepharose(^a)</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>Di-(6-aminocaproyl)-PTA</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>Di-(6-aminocaproyl)-PTA-Sepharose(^b)</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>

\(^{a}\) \( K_I \) 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.
The per cent yield is expressed relative to the applied crude enzyme solution, which had a specific activity of 25 to 32 and an activity defined as 100%.

Di-(6-aminocaproyl)-PTA, 560-610 65-75
Di-(6-aminocaproyl)-PTA, 520-575 65-75
Mono-(6-aminocaproyl)-PTA, 380-420 40-50

The quantity of crude enzyme added (0.7 mmoles per min per pmole 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 elutions. Nonlinear salt gradients gave generally better resolutions, and recoveries from these column runs were always in excess of 60%.

The larger preparative affinity column purification shown in Fig. 2 again uses the di-(6-aminocaproyl)-PTA gel, but here the elution of retained protein is accomplished with a nonlinear potassium chloride gradient and gives a much better resolution of enzyme from contaminating protein. A complete purification is not achieved in this run, however, as the specific activities of eluted enzyme are somewhat variable and correspond to about 85% of that of enzyme purified to the highest attainable specific activity. This column had been loaded with an excess of crude enzyme solution, and the enzyme activity capacity of the column was observed to be 0.7 mmoles per min per pmole of ligand. This corresponds to about 1 enzyme molecule bound per 400 ligand sites.

A summary of the results of affinity chromatography using di-(6-aminocaproyl)-PTA-Sepharose is given in Table III. These results were obtained with gels having either 0.71 or 1.61 μmoles of ligand per ml of packed Sepharose and will be shown below to indicate the isolation of essentially pure enzyme. Purification levels nearly as high were found by means of gels with a higher concentration of attached di-(6-aminocaproyl)-PTA; but such gels retain more contaminant protein, require higher concentrations of salt to elute the enzyme, and reduce yields because of their higher capacity to retain enzyme irreversibly.

The affinity chromatography results obtained with the use of an alternate ligand, mono-(6-aminocaproyl)-PTA, are also included in Table III. Lower levels of purification are attained with this ligand, which does not extend as far from the Sepharose polysaccharide backbone as di-(6-aminocaproyl)-PTA. The salt gradient elution of retained protein contaminants from mono-(6-aminocaproyl)-PTA-Sepharose overlaps that of the enzyme and decreases the resolution and purification from that obtained with the di-(6-aminocaproyl)-PTA gel. This difference is consistent with the somewhat higher affinity of the enzyme for di-(6-aminocaproyl)-PTA-Sepharose shown in Table II.

**Standard Chromatography**—The results obtained by standard chromatographic procedures are outlined in Table IV. Purification levels higher than those reported previously (5, 6) were observed in the first five steps, but the specific activities obtained in the last three steps are somewhat lower. The enzyme from Step 8 appears very close to being chromatographically pure; no protein is eluted in fractions separate from the active peak, and the specific activities of fractions on the tails of this peak remain above 450. 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 displaced enzyme could be rechromatographed on B-DEAE-cel-
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) 30 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.

Affinity Chromatography 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.
and their derivatives with free or potentially free reducing groups.

...protein contained significantly less than the 16.0% nitrogen as-...tions of NH₃ or N₄ are underestimating the protein content from the dry weight and refractive index. It appears that...Techniques for measuring absolute protein content were em-...tern with the protein estimated from the dry weight or the...dictations, as observed to be 3.52 ± 0.05. The corresponding ratio (ε⁺₂₈₀nm/ε⁺₃₆₀nm) was 2.1 ± 0.1. Conversion of nitrogen to protein was made assuming that the protein contained 16.0% nitrogen.

**Immuno-electrophoresis**—Patterns obtained at various stages of enzyme purification are shown in Fig. 5. While antiserum to crude enzyme measurably precipitated only protein impurities, antiserum 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 to 560, 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 the protein content from the dry weight and the refractive index. It appears that micro determinations of NH₃ or N₄ are underestimating the protein content by 10 to 15%. The difference could be real or 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-(6-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 for samples highly purified either by affinity or by standard chromatographic procedures and to be 3.52 ± 0.05. The corresponding ratio (ε⁺₂₈₀nm/ε⁺₃₆₀nm) was 2.1 ± 0.1. Conversion of nitrogen to protein was made assuming that the protein contained 16.0% nitrogen.

### Table V

#### Extinction coefficients based on several methods of absolute protein determination

<table>
<thead>
<tr>
<th>Protein determination</th>
<th>Number of determinations</th>
<th>ε⁺₂₈₀nm*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Analysis of nitrogen content by the Dumas or micro-Kjeldahl method</td>
<td>4</td>
<td>21.4 ± 0.3</td>
</tr>
<tr>
<td>2. Analysis of nitrogen content by ninhydrin micro-method</td>
<td>4</td>
<td>21.8 ± 0.7</td>
</tr>
<tr>
<td>3. Analysis of nitrogen content from the amino acid composition</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>

* 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 Δ(ε⁺₂₈₀nm − ε⁺₃₆₀nm) may also be used to estimate the protein concentration. The ratio of this difference extinction coefficient to ε⁺₂₈₀nm was very reproducible for samples highly purified either by affinity or by standard chromatographic procedures and was observed to be 3.52 ± 0.05.

### Table VI

#### Amino acid composition of acetylcholinesterase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per 12 valine residues*</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>Alanine</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>5.8</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>6.0</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></td>
</tr>
</tbody>
</table>

* Data are tabulated in terms of valine, which showed a constant mole per cent based on total amino acid content of 7.05 ± 0.05. 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 enzyme maximum velocity (13) is about 4% higher.

The turnover at the extrap-olated enzyme maximum velocity (13) is about 4% higher.

The enzyme turnover number per site, obtained directly from the enzyme-catalyzed acetylcholine hydrolysis and, if the molarit-y of the enzyme solution can be established, the number of enzyme catalytic sites per molecule. To determine the enzyme molar-ity 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 sedi-mentation 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 730 for enzyme purified by the standard chromatographic procedures; but a more recent report by Leu-zinger and Baker (6) report a specific activity of 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, $K_D$, observed for the complex of Bis-Q with enzyme was $5.4 \pm 0.5 \mu M$, and complex formation was completely reversible. This $K_D$ agrees with the competitive inhibition con-stant $K_I$ obtained with Bis-Q (Table II) and supports the conclusion that the Bis-Q binding observed occurs at the enzyme active site.

**TABLE VII**

<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>$7.66 \pm 0.25 \times 10^5$</td>
<td>$2.85 \pm 0.13$</td>
<td>$3.44 \pm 0.21$</td>
</tr>
<tr>
<td>M7C2</td>
<td>$8.03 \pm 0.26 \times 10^5$</td>
<td>$3.15 \pm 0.15$</td>
<td>$3.28 \pm 0.20$</td>
</tr>
<tr>
<td>Bis-Q</td>
<td>$7.63 \pm 0.59 \times 10^5$</td>
<td>$2.92 \pm 0.24$</td>
<td>$3.86 \pm 0.31$</td>
</tr>
<tr>
<td>Bis-Q2</td>
<td>$8.80 \pm 0.53 \times 10^5$</td>
<td>$2.81 \pm 0.21$</td>
<td>$2.98 \pm 0.25$</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 505 ± 15; the protein concentra-tion was $14.3 \pm 0.19 \mu g$ per ml; and the observed normality was $1.58 \pm 0.03 \times 10^{-7} N$.

Enzyme specific activity was 558 ± 10; the protein concentra-tion was $25.9 \pm 0.55 \mu g$ per ml; and the observed normality was $3.14 \pm 0.05 \times 10^{-7} N$.

Enzyme specific activity was 515 ± 15; the protein concentra-tion was $917 \pm 27 \mu g$ per ml; and the observed normality was $103.1 \pm 6.9 \times 10^{-7} N$.

Enzyme specific activity was 575 ± 15; the protein concentra-tion was $90 \pm 19 \mu g$ per ml; and the observed normality was $102.6 \pm 6.1 \times 10^{-7} N$.

An attractive feature of the use of M7C as a titrating agent is that the fact that the entire course of the carbamoylation reaction may be observed directly. Hence the following kinetic param-eters, rigorously defined in a previous paper (13), were observed for the titration reaction: the specific carbamoylation rate con-stant, $k_b$, was $5.1 \pm 0.8 \text{ min}^{-1}$; the specific decarbamoylation rate constant, $k_a$, was $0.033 \pm 0.010 \text{ min}^{-1}$; and the pre-car-bamoylation (reversible) titrant dissociation constant, $K_a$, was $6.7 \pm 1.8 \mu M$. 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.

**DISCUSSION**

A specific activity of 660 for acetylcholinesterase highly puri-fied by the standard chromatographic procedures (Table IV) was observed by Kremer and Wilson (5). However, relative ultraviolet absorption values given for this preparation ($\epsilon_{280} \text{ nm} = 22.9; \Delta (\epsilon_{280} \text{ nm} - \epsilon_{340} \text{ nm})/\epsilon_{340} \text{ nm} = 2.78; \epsilon_{280} \text{ nm}/\epsilon_{340} \text{ nm} = 1.67 (7)$) 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 Bakor (6) report a specific activity of 730 for enzyme purified by the standard chromatographic procedures; but a more recent report by Leu-zinger (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 discomfiting 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. Valee Harris-dangkul to the immunoelectrophoresis and nitrogen determination results and by Dr. William Poillon to the differential refractometry measurement.

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