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GUINEA PIG BRAIN ACETYLCHOLINESTERASE: PARTIAL PURIFICATION BY AFFINITY CHROMATOGRAPHY*

HENRY I. YAMAMURA, DOUGLAS W. REICHARD, TOMMY L. GARDNER, JOEL D. MORRISETT** AND CLARENCE A. BROOMFIELD

Medical Research Division, Biomedical Laboratory, Edgewood Arsenal, Md. 21010 (U.S.A.)

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SUMMARY

The technique of affinity chromatography was used in the partial purification of guinea pig brain acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7). The enzyme from brain homogenates was solubilized by treatment with Triton X-100. 80% solubilization of the enzyme was achieved. The solubilized enzyme was passed through a small column of aminobutyl-Sepharose to which specific competitive inhibitors had been covalently attached. A high percentage of the nonspecific protein passed directly through the affinity column while the specific enzymatic protein remained bound to the gel. NaCl and choline chloride were used as eluants to release the bound enzymatic activity from the affinity support. *m*-Carboxyphenyl trimethylammonium iodide attached through 1,4-diaminobutane to Sepharose 4B proved to be more efficient in binding the enzymatic protein than its *p*-analog. A linear gradient elution utilizing choline chloride preceded by a NaCl wash proved to result in a rapid and efficient means of releasing the bound enzymatic activity. The results of the study showed that a specific activity of at least 10 mmoles of [^{14}C]acetylcholine hydrolyzed/h per mg protein could be achieved, a 1000-fold purification. The partially purified enzyme had an apparent K_m of $1.47 \cdot 10^{-4}$ M using [^{14}C]acetylcholine as the substrate.

INTRODUCTION

Solubilization of brain acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is necessary before any purification steps can be taken because most of the enzyme has been found to be associated with subcellular particulates[1]. The same is true for acetylcholinesterase obtained from other organs. The solubilization of heart cholinesterase by trypsin hydrolysis has been reported by Ord and Thompson[2]. Jackson and

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** Present address: Department of Internal Medicine, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77025.

Aprison[3,4] have solubilized beef brain acetylcholinesterase by treating the anhydrous dried powdered enzyme with *n*-butanol and anhydrous diethyl ether. Lawler[1] has solubilized the brain enzyme by treating the sonicated suspension of brain with lipase. Kaplay and Jagannathan[5] have obtained a 50-fold purification of brain acetylcholinesterase by solubilizing with pancreatic elastase.

There have been several reports of the use of a nonionic detergent, Triton X-100, to solubilize the enzyme. Kremzner *et al.*[6] have used the nonionic detergent for preparation of acetylcholinesterase from human caudate nuclei. Recently, Ho and Ellman[7] and Crone[8] have reported on the solubilization of acetylcholinesterase from rat brain by Triton X-100. Because of these successful results we used Triton X-100 to solubilize the enzyme from the guinea pig brain using a method similar to that of Ho and Ellman[7].

We (Broomfield *et al.*[9]), as well as others[10–12] have recently reported on the use of affinity chromatography in the purification of acetylcholinesterase from the electric tissue of *Electrophorus electricus*. The principle of affinity chromatography recently has found wide application due mainly to the work of Cuatrecasas and associates[13–15] with derivatized agarose compounds, although chromatographic techniques utilizing the specificity of a ligand-protein reaction have been employed on previous occasions[16–18].

In this communication, we wish to report on the selective binding of acetylcholinesterase from solubilized guinea pig brain tissue to *p*-carboxyphenyl trimethylammonium iodide and *m*-carboxyphenyl trimethylammonium iodide, which were covalently bound to aminobutyl-Sepharose 4B. The present method is simple and a high level of enzyme purification can be achieved.

MATERIALS AND METHODS

[^{14}C]Acetyl-β-methylcholine iodide (2.35 Ci/mole) and [^{14}C]acetylcholine iodide (2.4 Ci/mole) were purchased from the New England Nuclear Corp., Boston, Mass. Amberlite CG-120 resin, (sodium form, 200–400 mesh analytical grade) was a product of the Mallinckrodt Co., St. Louis, Mo. Naphthalene was obtained from Eastman Organic Chemicals, Rochester, N.Y. 2-5Diphenyloxazole (PPO) and 1,4-bis-(2-5-phenyloxazolyl)-benzene (POPOP) were obtained from Packard Instruments Co., Downers Grove, Ill. Lubrol WX was obtained from I.C.I. Organics Incorporated, Stamford, Conn. Choline chloride was obtained from Eastman Kodak Co., Rochester, N.Y. Triton X-100 was from Rohm and Hass Co., Philadelphia, Pa. Sepharose 4B was obtained from the Pharmacia Co., Sweden.

The radiometric assay for acetylcholinesterase activity was used[19]. The method is based upon the removal of unreacted [^{14}C]acetyl-β-methylcholine with Amberlite CG-120 resin suspended in dioxane. The supernatant solution containing the product of hydrolysis, the free [1-^{14}C]acetic acid, is counted in a Packard Tricarb liquid scintillation spectrometer, model No. 3375 (66% efficiency). Details concerning preparation of resin and the scintillation cocktail are given by Siakotos *et al.*[19].

Preparation of a standard curve relating radioactivity to hydrolysis of substrate

30.5 mg of [^{14}C]acetyl-β-methylcholine was dissolved in 1 mM acetate buffer at pH 4.5. Sufficient nonlabeled acetyl-β-methylcholine was added to a given concentra-

tion of 0.003 mmole/ml ($3 \cdot 10^{-3}$ M). This stock solution was subdivided and stored in polyethylene vials at −20 °C. No significant occurrence of loss in activity was detected over a six month interval when stored under these conditions. A similar procedure was used for the preparation of [^{14}C]acetylcholine.

For preparation of a standard for assay of acetylcholinesterase activity of brain tissue, the stock was diluted with dioxane to contain 15 nmoles/ml. Aliquots of this dilution, varying from 0.1–5 ml with the difference in volume compensated with pure dioxane, were added to 12 ml of Bray's cocktail. The samples were counted in a scintillation spectrometer, with an efficiency of 66% at the optimized ^{14}C setting. The radioactivity in cpm was plotted against the nmoles of substrate. After removal of unhydrolyzed substrate with dioxane resin, the radioactivity in 5 ml of dioxane supernatant was measured, and the acetylcholinesterase activity estimated by interpolation.

Preparation of brain tissue and enzyme assay

Adult guinea pigs (Hartley strain) of either sex and weighing between 300–500 g were killed by decapitation. The whole brain from each animal was rapidly removed, rinsed in ice-cold 0.9% saline, blotted and weighed. 5% homogenates of the whole brain were prepared in 0.1 M sodium phosphate buffer (pH 8.0) by making 10 strokes in an all glass homogenizing tube. The homogenate was transferred to capped centrifuge tubes and ultracentrifuged at 100 000 × g (Beckman ultracentrifuge, No. 30 rotor) for 60 min at 4 °C. The supernatant was decanted and usually discarded. The pellet was then resuspended in an appropriate volume of ice-cold 0.1 M sodium phosphate buffer at pH 8.0 and rehomogenized. To the reconstituted pellet was added Triton X-100 (0.7%). After thoroughly mixing on a Vortex vibrator, the contents of the tube were incubated at 37 °C for 30 min with periodic shaking. The reconstituted pellet was then centrifuged at 100 000 × g for 90 min at 4 °C, and the clear supernatant was decanted from the small amount of white matter found at the bottom of the centrifuge tube. This supernatant formed the standard Triton-solubilized preparation.

Aliquot samples were assayed for acetylcholinesterase activity by adding 100 μl of sample to 100 μl of 0.1 M sodium phosphate buffer at pH 7.8 containing 0.3 M NaCl and 1% Lubrol WX. 0.1 ml of 0.003 M [^{14}C]acetyl-β-methylcholine or [^{14}C]acetylcholine was then added and the samples were mixed and incubated for various time intervals at 37 °C. The reaction was immediately stopped by the addition of dioxane-resin. After mixing and centrifuging, a 5-ml aliquot of the supernatant was taken and counted in a liquid scintillation spectrometer.

Protein

Protein was determined by the method of Lowry *et al.*[20]. Precipitates in some samples, due to the presence of Triton X-100, were removed by centrifugation in an International refrigerated centrifuge, model No. PR-6 before reading the absorbance.

Preparation of the column material

The preparation of the column material was essentially done according to the procedure described by Cuatrecasas[21].

In a well ventilated hood, 100 ml of washed and settled Sepharose 4B was mixed

with 100 ml of a water solution containing 10 g of cyanogen bromide. The pH was immediately raised to, and maintained at, 10 with 8 M NaOH. The reaction was essentially complete in 30 min, as indicated by the cessation of proton release. A large amount of ice (approx. 200 ml) was then rapidly added to the suspension, which was transferred quickly to a Buchner funnel (coarse disc) and washed under suction with cold buffer (0.1 M Na_2CO_3, pH 10.0) using approx. 2 l. The buffer was the same as that which was to be used in the coupling stage (see below) and the volume of the wash was 10 to 15 times that of the packed Sepharose. The washed Sepharose was then quickly transferred to a beaker containing a ligand to be coupled (17.6 g of 1,4-diaminobutane) in a volume (100 ml, pH 10.0) of cold buffer equal to volume of packed Sepharose. It was important that these procedures be performed rapidly and that the temperature be lowered, since the activated Sepharose was unstable. The suspension was then gently stirred at 4 °C. Although the reaction was essentially complete in 2–3 h, the mixture was allowed to stand at 4 °C for 16 to 20 h to insure complete loss of reactive groups. The substituted Sepharose was then washed with large volumes of water to remove all excess ligand.

Coupling reaction

To 50 ml of aminobutyl-Sepharose in 50 ml of water was added 1.2 g (4.3 mmoles) of *p*- or *m*-carboxyphenyl trimethylammonium iodide dissolved in 100 ml of water and the pH adjusted to 4.75. Then a solution of 2.46 g (12.9 mmoles) of 1-dimethylaminopropyl-3-ethylcarbodiimide·HCl dissolved in 100 ml of water and adjusted to pH 4.75 was added slowly over a 10-min period. During the addition of the coupling reagent, the pH was maintained at 4.75 by the addition of 0.1 M HCl. This was maintained for several hours at which time the reaction was left unstirred and unmonitored overnight.

The theoretical capacity of column material produced in the above process may be estimated by measuring the acid uptake during the coupling reaction. However, the theoretical capacity is not necessarily related to the practical capacity, which consistently seems to be much lower than would be predicted theoretically. For example, 50 ml of settled Sepharose 4B consumes approx. 3 mmoles of acid. However, this material begins to reject eel acetylcholinesterase activity after approximately 10^{-3} mmole of the enzyme had bound.

RESULTS

Brain acetylcholinesterase solubilized by Triton X-100

A pellet was obtained from the original membrane bound enzyme by homogenization and centrifugation (100 000 × *g* for 60 min at 4 °C). The pellet was then reconstituted with buffer containing 0.7% Triton X-100. Incubation of the reconstituted pellet was carried out at 37 °C for 30 min with periodic shaking on the Vortex vibrator. Following incubation, the mixture was again centrifuged at 100 000 × *g* at 4 °C for 90 min.

Experiments showed that treatment with Triton X-100 and ultracentrifugation transferred approx. 80% of the total acetylcholinesterase activity from the reconstituted pellet (405.7 ± 21.8 μmoles [^{14}C]acetyl-β-methylcholine/h) into the supernatant fraction (308 ± 21.3 μmoles [^{14}C]acetyl-β-methylcholine/h). The supernatant

having a specific activity 2-fold higher than the original 5% homogenate was used as the solubilized enzyme source which was applied to the affinity column.

Affinity chromatography of triton solubilized enzyme

The effect of eluants on the release of acetylcholinesterase from the "p-quat" column. In the experiment illustrated in Fig. 1, a small column (1 cm × 15 cm) containing aminobutyl-Sepharose 4B, to which a specific competitive inhibitor (*p*-carboxyphenyl trimethylammonium iodide, *p*-quat) had been covalently attached, was used. It was felt that the enzyme would probably be sterically inhibited from binding by the proximity of the agarose chain if an inhibitor were attached directly to the hydroxyl groups of agarose[13]. Therefore, the sidechains were first extended by coupling putres-

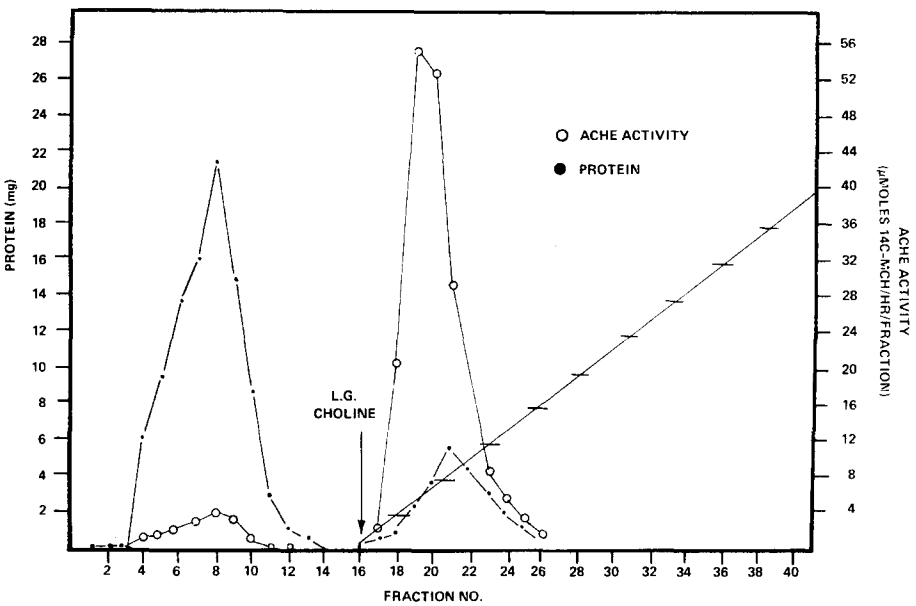

Fig. 1. Elution of acetylcholinesterase (ACHE) from the "*p*-quat" column with a continuous linear gradient (L.G.) of choline chloride (0 to 1.0 M). Crude enzyme solution with a total activity of 13.9 μmoles [^{14}C]acetyl-β-methylcholine (MCH)/h (7.0 mg protein) was passed through the column and 4.0-ml fractions of eluant were collected every 15 min.

cine (1,4-diaminobutane) to the agarose, and then coupling the inhibitor to the free amino groups with a water-soluble carbodiimide, as stated in the Materials and Methods section. Columns were prepared using Sepharoses 2B, 4B, and 6B with the *p*-quat inhibitor; however, only results from the Sepharose 4B column are reported here since the resolution of enzyme activity from contaminants was best observed with this column.

After equilibration of the affinity column with phosphate buffer of low ionic strength, pH 7.4 (0.05 M NaCl–0.01 M phosphate), enzyme with an activity of 13.9 μmoles [^{14}C]acetyl-β-methylcholine/h was applied to the column and the gel was washed with sodium phosphate buffer of low ionic strength until no further evidence

of protein or enzyme activity appeared in the effluent. Approx. 90% of the total, nonspecific protein passed directly through the column as represented by the contents of tubes 1 through 14 (see Fig. 1). By measuring the total enzyme activity, it was determined that most of the acetylcholinesterase activity (90%) remained bound to the gel. Thus, 10% of the original crude protein, containing 90% of the total enzyme activity, remained firmly bound to the column. Choline chloride (linear gradient, 0 to 1 M) was then used to elute the bound enzyme from the column, as seen in tubes 16 through 40.

Specific enzyme activity, determined after complete dialysis of choline chloride from the content of the peak tube (Fraction No. 19), was 22 μmoles [^{14}C]acetyl-β-methylcholine/h per mg protein or a 10-fold increase in purity over the original supernatant (2.08 μmoles [^{14}C]acetyl-β-methylcholine/h per mg protein). The finding that an enzyme activity peak appeared at 0.12 M choline chloride while the protein peak occurred at a concentration of 0.2 M choline chloride indicated the binding of contaminating protein to the gel, resulting in less purification.

Fig. 2 illustrates the results obtained when NaCl was used as the eluant. A solubilized enzyme solution (12.4 μmoles [^{14}C]acetyl-β-methylcholine/h) obtained by reconstituting the pellet as previously described was applied to the affinity column. The column was washed with sodium phosphate buffer of low ionic strength (0.05 M NaCl–0.01 M phosphate) followed by elution with a linear gradient (0 to 1 M) of NaCl. A protein peak appeared at 0.2 M NaCl, as in Fig. 1 when choline chloride was used. However, the enzymatic activity peak (Fraction No. 26) here was eluted after the protein peak resulting in a specific activity of 64 μmoles [^{14}C]acetyl-β-methylcholine/h

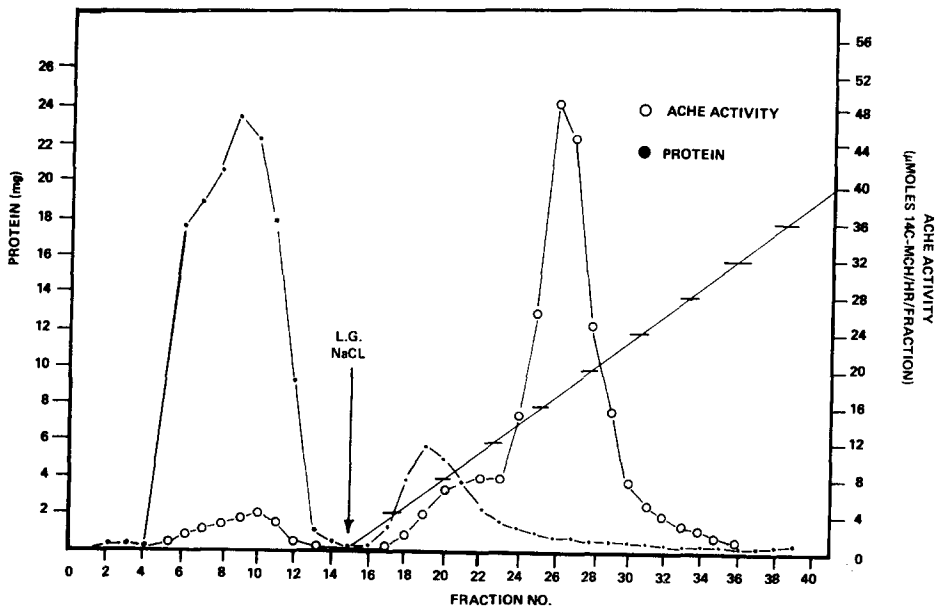

Fig. 2. Elution of acetylcholinesterase (ACHE) from the "*p*-quat" column with a continuous linear gradient (L.G.) of NaCl (0 to 1.0 M). Crude enzyme solution with a total activity of 12.4 μmoles [^{14}C]acetyl-β-methylcholine (MCH) /h/7.0 mg protein was passed through the column and 4.0-ml fractions of sample were collected every 15 min.

per mg protein, a 36-fold purification of the enzyme. This difference in the elution profiles with choline chloride and a neutral salt was predicted for an affinity chromatography technique.

Attempts were made to eliminate more of the nonenzymatic protein by first eluting with NaCl, then applying a linear choline chloride gradient. It was hoped that greater purification of the enzyme would be achieved by this procedure.

Four guinea pig brain tissues were solubilized separately and 16.2 μmoles [^{14}C]acetyl-β-methylcholine/h of enzyme activity was applied to the column. The column was then washed with sodium phosphate buffer of low ionic strength as described previously. The NaCl concentration that would remove as much contaminating protein as possible without elution of bound enzymatic protein was determined in preliminary experiments. The appropriate salt concentration, approx. 0.2 M, was then applied to the column and fractions were collected as described previously. The column was then again washed with the low salt buffer.

Finally, choline chloride (linear gradient 0 to 0.5 M) was used to release the bound enzymatic protein. Analysis of the contents of the peak tube revealed a specific activity of 548 μmoles [^{14}C]acetyl-β-methylcholine hydrolyzed/h per mg protein, or greater than a 200-fold purification of the enzyme.

The effect of eluants on the release of acetylcholinesterase from the "m-quat" column. In the course of the investigation, modifications to the active portion of the column were made. In addition to the *p*-quat, an additional analog was synthesized (*m*-carboxyphenyl trimethylammonium iodide, *m*-quat). An experiment similar to that shown in Fig. 1 for the *p*-quat column was performed using the *m*-quat gel. The results are illustrated in Fig. 3. It was observed that more than 95% of the total protein from one solubilized guinea pig brain tissue passed directly through the *m*-quat column. Elution with a linear choline chloride gradient (0 to 0.2 M) produced a peak tube (Fraction No. 18) with a specific activity of 106 μmoles [^{14}C]acetyl-β-methylcholine/h per mg protein, in contrast to 22 μmoles [^{14}C]acetyl-β-methylcholine/h per mg protein found in the content of the peak tube (Fraction No. 19) with the *p*-quat column. Of importance was the finding that very little protein and enzyme activity were released with high concentrations of NaCl (1 M) at the end of the experiment in Fig. 3 (Fraction Nos 34–36). This indicated a good recovery of both bound protein and enzyme from the affinity column.

In an attempt to achieve greater enzyme purification with the *m*-quat column, an experiment was performed in which ten times the volume of the previous experiment was loaded onto the column. In this experiment, ten guinea pig brain tissues were solubilized separately and applied separately onto the column. Since we were primarily interested in bound enzymatic protein, it was decided to collect portions of the effluent containing unbound nonspecific protein in a large flask and carry out the analysis for protein and enzyme activity after recording the total volume collected. After application of the solubilized enzyme preparation and subsequent washing with low ionic strength buffer, a solution of NaCl (0.2 M) was applied to the column in an attempt to elute most of the contaminating protein. This latter effluent was also collected separately in a large flask and subsequently analyzed as bulk protein and enzyme activity. Elution with choline chloride (linear gradient, 0 to 0.2 M) was then performed.

Although approximately the same amount of enzyme, but ten times the volume

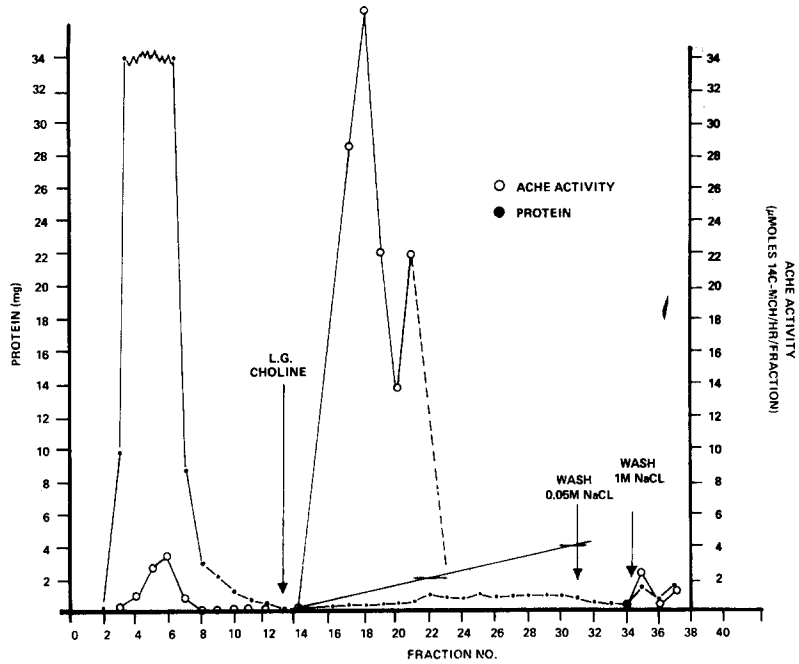

Fig. 3. Elution of acetylcholinesterase (ACHE) from the "*m*-quat" column with a continuous linear gradient (L.G.) of choline chloride (0 to 0.2 M). Crude enzyme solution with a total activity of 20.7 μmoles [^{14}C]acetyl-β-methylcholine (MCH)/h (9.0 mg protein) was passed through the column and 5.5-ml fractions of sample were collected every 15 min.

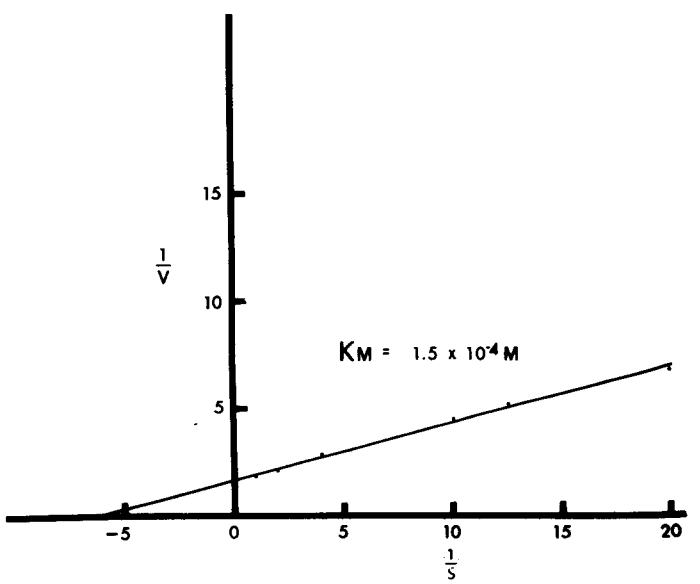

Fig. 4. Kinetic analysis of the affinity of [^{14}C]acetylcholine for purified guinea pig brain acetylcholinesterase using the Lineweaver–Burk technique (each point is an average of at least 8 determinations). The purified enzyme had an apparent K_m of $1.5 \cdot 10^{-4}$ M using [^{14}C]acetylcholine as the substrate.

was loaded onto the column, only 67% of the enzyme activity was bound to the *m*-quat column. The application of NaCl (0.2 M) resulted in elution of approx. 30% of the enzyme activity and about 1% of the contaminating protein. Elution with choline chloride (linear gradient, 0 to 0.2 M) resulted in an enzyme activity with very high specific activity. Calculations showed that a specific activity of at least 10 mmoles of [^{14}C]acetylcholine hydrolyzed/h per mg protein was achieved in the contents of the peak tube, a 1000-fold purification of the enzyme. The ratio of hydrolysis of [^{14}C]acetylcholine to [^{14}C]acetyl-β-methylcholine determined radiometrically was 5.21 ± 0.2 ($n = 5$).

Kinetic constant of the Triton X-100 solubilized enzyme

The affinity of the substrate for the partially purified enzyme was determined at seven different concentrations (Fig. 4) of [^{14}C]acetylcholine ranging from 0.05 to 1.0 mM. From a Lineweaver–Burk plot, the K_m value was determined to be $1.47 \cdot 10^{-4}$ M.

DISCUSSION

Attempts by others[1,4] to purify acetylcholinesterase from brain tissue have met with only partial success, even when complicated and time consuming separatory procedures have been employed[7]. The present steps employed in the solubilization and partial purification are simple and reliable for obtaining the enzyme on a small scale from guinea pig brain.

Methods for solubilization of acetylcholinesterase have been reported[2,4,6]. More recently, the actions of a nonionic detergent, Triton X-100 on the enzyme obtained from the rat brain have been studied[7,8]. Accordingly, treatment of guinea pig homogenates with Triton X-100 has proven to be quite effective in solubilizing brain acetylcholinesterase. Approx. 80% of the enzyme activity was found in the supernatant fraction after incubation with the nonionic detergent and ultracentrifugation under the conditions of the present study.

In recent years, the technique of affinity chromatography[13–15] has been exploited in the purification of proteins whose biological activity could not withstand the more conventional chromatographic methods. In this technique, a substrate-analog inhibitor is covalently attached to a suitable matrix thereby obtaining a solid support capable of binding specifically and reversibly the enzyme of interest.

We have performed comparative studies using aminobutyl-Sepharose 4B as the backbone matrix to which were coupled the following specific competitive inhibitors: *p*-carboxyphenyl trimethylammonium iodide and *m*-carboxyphenyl trimethylammonium iodide. Eluants were used alone and in combinations for the elution of the bound enzymatic activity from the column. The position of the enzyme activity peak relative to the protein impurity peak indicates that both of the columns are functioning as true affinity supports. When either column is eluted with a salt gradient, the enzyme activity is eluted after the protein impurities, because it is bound more strongly by virtue of its specific, active-site binding. When elution is carried out with choline chloride, on the other hand, the enzyme activity precedes the protein impurities because the inhibitor competes for the specific active-site binding and at lower concentrations contributes very little to nonspecific, ion-exchange type elution.

NaCl (0.2 M), followed by a linear choline chloride gradient (0 to 0.5 M), proved to be quite effective for the elution of the enzyme from the *p*-quat column. Purification of the enzyme to a specific activity of approx. 0.5 mmole of [^{14}C]acetyl-β-methylcholine hydrolyzed/h per mg protein was achieved, a 200-fold purification.

In the utilization of the *m*-quat column, further enrichment of the enzymatic activity resulted. Of significance was the finding that a majority of the nonspecific or contaminating protein was not bound to the column indicating its enhanced specificity for the enzymatic protein. Attempts to obtain greater enzyme purification by loading of larger volumes of crude enzyme preparation did not result in correspondingly greater adsorption of binding activity. Thus, we found only 67% of the enzyme activity bound when ten times the volume was applied to the column. This poorer binding of active enzyme was probably due to competition for the binding sites by the large amounts of crude, concentrated protein applied which prevented binding of the smaller quantity of enzymatic protein present. This finding was also observed by Kalderon *et al.*[10], while attempting to further purify acetylcholinesterase from the electric eel and by Cuatrecasas[22] while attempting to purify insulin receptor of liver cell membranes. Even with lower percentages of enzyme activity bound, a specific activity of 10 mmoles of [^{14}C]acetylcholine hydrolyzed/h per mg protein was achieved, a 1000-fold enrichment, after a NaCl (0.2 M) elution followed by a linear choline chloride gradient (0 to 0.2 M). The enzyme from the contents of the peak tube was analyzed kinetically for its affinity for acetylcholine. From a Lineweaver–Burk plot, the enzyme had an apparent K_m value of $1.47 \cdot 10^{-4}$ M.

Although direct comparisons cannot be made with others due to their use of different substrates, Jackson and Aprison[4] reported that their purified beef brain enzyme hydrolyzed 1.11 mmoles of acetylthiocholine/h per mg protein with a K_m value of $1.38 \cdot 10^{-4}$ M while Ho and Ellman[7] reported that their rat brain acetylcholinesterase hydrolyzed 0.05 mmole of acetylthiocholine/h per mg protein after purification.

The application of affinity chromatography in the partial purification of brain acetylcholinesterase, as reported in this study, appears to be relatively simple. The single step chromatographic procedure will readily enhance the availability of the enzyme for more detailed biochemical studies in the future.

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