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ON THE HOMOGENEITY OF 11-S ACETYLCHOLINESTERASE

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Summary

11-S acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) purified by affinity chromatography of trypsin-digested homogenates was shown to be contaminated with three other active forms of the enzyme. The initial purification used an affinity column of the inhibitor, *N*-methylacridinium ion. Chromatography of the "affinity-pure" sample on hydroxyapatite resulted in two peaks of acetylcholinesterase activity. One peak contained only a form sedimenting at 11-S (approx. 85% of the recovered activity). The other peak consisted of a 9.5-S form, in addition to 14-S and 18-S forms. The 9.5-S form (approx. 7% of the activity) co-electrophoresed with 11-S in 6% polyacrylamide gels and co-sedimented with the same form in sucrose density gradients containing 0.1 M NaCl. The purified 11-S enzyme was shown to be homogeneous by sucrose density gradient centrifugation and electrophoresis. These results indicate that 11-S acetylcholinesterase may be unsuitable for some characterization studies due to undetected contamination by the 9.5-S form.

Introduction

Considerable attention has been given to the isolation of a homogeneous preparation of the most stable form of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), the globular 11-S tetramer.

In recent years, affinity chromatography using covalent or non-covalent ligands has become the preferred method for isolation of the various forms of acetylcholinesterase from crude extracts of electroplax tissue of the electric eel (*Electrophorus electricus*) [1—6]. Phenyltrialkylammonium columns have been

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used primarily for purification of 11-S only, whereas *N*-methylacridinium columns have been introduced for isolation of the high molecular weight forms (14 and 18-S), in addition to the 11-S form.

The affinity-purified 11-S acetylcholinesterase of early studies was shown to be heterogeneous by polyacrylamide gel electrophoresis, and this heterogeneity was attributed to higher molecular weight forms of the enzyme. Dudai et al. [7] used the trypsin digest technique of Massoulié et al. [8,9] and purified an essentially homogeneous 11-S form. Rosenberry et al. [2] have purified acetylcholinesterase from a digest that was not trypsin-treated, and this was shown to contain an inactive impurity (10% of the protein) and also some material that did not pass through a spacer gel. We report here our results on the isolation of homogeneous 11-S acetylcholinesterase from tryptic digests by affinity and hydroxyapatite chromatography.

Experimental

Materials. Electric eels (*E. electricus*) were purchased from Paramount Research Supply Co., (Ardsley, N.Y.). Sepharose 4B was obtained from Pharmacia (Piscataway, N.J.) and hydroxyapatite (Bio-Gel HT) from Bio-Rad (Rockville Centre, N.Y.). Acetylthiocholine iodide and 5,5′-dithiobis-(2-nitrobenzoic acid) were supplied by Calbiochem (Baltimore, Md.). CNBr, 1,2-diaminopropane, *N*-benzyloxycarbonyl-ϵ-aminocaproic acid, acrylamide, and *N,N′*-methylenebisacrylamide were obtained from Aldrich (Metuchen, N.J.). CH_3I and 9-chloroacridine were supplied by Fisher (Silver Spring, Md.) and decamethonium bromide by Pfaltz and Bauer (Stamford, Conn.). Ultrafiltration equipment was provided by Amicon (Lexington, Mass.). Trypsin, soybean trypsin inhibitor, catalase, and lactate dehydrogenase were purchased from Worthington (Freehold, N.J.); β-galactosidase, phosphorylase a, and ovalbumin from Sigma (St. Louis, Mo.); and bovine serum albumin from Pentex (Kankakee, Ill.).

Estimation of protein concentration and enzyme activity. Protein was estimated at 280 nm using the value of $A_{280}^{1\%} = 18$ [2]. Enzyme activity was measured by using the colorimetric procedure of Ellman et al. [10]. An activity of one unit is equivalent to 1 ΔA_{412}/min.

Synthesis of affinity ligand. 1-methyl-9-[(N^{β}-ϵ-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide (*N*-methylacridinium) was prepared by the method outlined below with particular emphasis on the preparation of 9-(β-aminopropylamino)acridine. Infra-red spectra were recorded with a Perkin-Elmer Model 21 double beam spectrophotometer. Mass spectral data were obtained on a Finnegan Model 3200 spectrometer at 70 eV and 1.0 mA emission via a solid probe programmed at 20°C/min from 25 to 250°C. Elemental analyses were provided by the microanalytical section of the National Institute of Arthritic and Metabolic Diseases, NIH, Bethesda, Md.

Synthesis of 9-(β-aminopropylamino)acridine (Compound IV) of Dudai and Silman [1]). 3.9 g 1,2-diaminopropane (52.5 mmol) mixed with 25 g anhydrous phenol under an N_2 flow was heated at 100°C for 15 min to remove surface moisture and then cooled to 40°C, 5 g 9-chloroacridine (23.5 mmol) was added and the temperature raised to 70°C for 30 min. After cooling to room

temperature, the mixture was transferred to 400 ml of ice-cold 1.5 M NaOH to dissolve the phenol and was stirred for 1 h. The phenolate solution was extracted twice with 100 ml $CHCl_3$. The combined extracts were washed 5 times with 50 ml H_2O (to remove unreacted diaminopropane) and then dried over anhydrous Na_2SO_4. Evaporation of the $CHCl_3$ at room temperature by a gentle stream of dry air yielded a crude product which was further dried overnight in vacuo over NaOH. Recrystallization from benzene yielded 4.1 g (63%) of yellow powder, m.p. 133—134°C. Analysis for $C_{16}H_{17}N_3$: calculated: C, 76.51; N, 16.72; found: C, 76.43; N, 16.91. Infra-red spectrum (KBr disc): 3323, 1625, 1518, 1260, 702 cm^{-1}. EI Mass Spectrum, m/e (rel. abund.): 251(18), 221(20), 207(100), 179(25), 151(15).

Synthesis of 1-methyl-9-[N^{β}-(N-benzyloxycarbonyl-ε-aminocaproyl)-β-aminopropylamine]acridinium iodide and N-methylacridiunium (Compounds VI and VII, respectively, of Dudai and Silman [1]). Synthesis of Compound VI and Compound VII followed the procedure of Dudai and Silman [1]. Analytical data available on request.

Preparation of affinity gel. Sepharose-4B was activated at 0—4°C by adding 85 ml 5 M potassium phosphate (pH 12.2) (3.35 mol K_2HPO_4 and 1.67 mol K_3PO_4/l.) to 100 ml settled gel beads and then adding 45 ml CNBr in H_2O (15 mg/ml gel) over a 2 min period. A total of 10 min (including addition) was allowed for reaction. The activated gel suspension was immediately filtered and washed with 1 l of cold 0.25 M $NaHCO_3$ (pH 9.5). Coupling was effected by adding the activated gel to 100 ml cold 0.25 M $NaHCO_3$ (pH 9.5) containing 1 μmol *N*-methylacridinium per ml gel. This suspension was agitated overnight in the cold by gentle shaking. After reaction, the coupled product was thoroughly washed with 10 mM sodium phosphate (pH 7.5)/1 M NaCl/20 mM $MgCl_2$/3 mM NaN_3 (Buffer A). The absorbance of all washings was monitored at 412 nm to determine unbound ligand; bound ligand was calculated by difference to be 0.8 μmol/ml gel. An affinity column (2.5 × 55 cm) was equilibrated with several volumes of Buffer A at 4°C.

Extraction. Acetylcholinesterase was extracted at 4°C from frozen electric organ tissue by homogenizing 300 g tissue with 500 ml of Buffer A. The homogenate was first incubated with 10 ml trypsin solution (10 mg/ml Buffer A) for 20 h at 25°C with stirring and then with 10 ml soybean trypsin inhibitor (15 mg/ml Buffer A) for 30 min at 25°C. The tryptic digest was centrifuged at 78 000 × *g* for 90 min at 4°C and the pellet discarded.

Affinity chromatography. Crude acetylcholinesterase (400 000 units) was applied to the affinity column at a flow rate of 15—20 ml/h. The column was then thoroughly flushed with at least 5 vols. Buffer A or until $A_{280} < 0.05$. The eluate was collected in 200—500 ml fractions and monitored for both A_{280} and activity. Enzyme was displaced from the column by eluting with Buffer A/20 mM decamethonium bromide. The eluate was collected in 10-ml fractions. Active fractions were pooled and dialyzed against 3 changes of 10 vols. each of Buffer A to remove decamethonium ion. The dialyzed solution was concentrated at 4°C under 20 lbs/in^2 N_2 using a Diaflo pressure cell with a PM 10 membrane. The concentrated solution was dialyzed against 10 vols. 10 mM sodium phosphate (pH 7.5)/0.1 M NaCl/10 mM $MgCl_2$/3 mM NaN_3.

Hydroxyapatite chromatography. A hydroxyapatite column (2.0 × 23 cm)

was equilibrated at room temperature with 10 mM sodium phosphate (pH 7.5)/ 0.1 M NaCl/3 mM NaN_3. Affinity-purified enzyme was then applied to the column and eluted with a linear 300 ml gradient of equal volumes of 0.01 M and 0.50 M sodium phosphate buffers (pH 7.5)/0.1 M NaCl/3 mM NaN_3. The eluate was collected in 3—4-ml fractions at 15 ml/h. Fractions were assayed for protein, activity, and sodium phosphate concentration [11]. Active fractions were pooled, concentrated under pressure, and dialyzed against 10 mM sodium phosphate (pH 7.5)/0.1 M NaCl/5 mM $MgCl_2$/3 mM NaN_3.

Estimation of sedimentation coefficients. Sucrose density gradient centrifugation was performed in a Spinco Model L2-75B centrifuge using a SW 50.1 Rotor. Catalase (bovine liver) and lactate dehydrogenase (beef heart) were used as markers with S values of 11.3 and 7.2, respectively [12]. Routinely, 50- or 100-μl samples were layered onto 5 ml cold linear sucrose gradients (5—20%) prepared with either 0.1 or 1 M NaCl in 10 mM sodium phosphate (pH 7.4). Samples were centrifuged at 33 000 rev./min for 16 h at 4°C. 150-μl fractions were collected.

Polyacrylamide gel electrophoresis. Electrophoresis was performed at 23°C in 6% gels at pH 9.0 with a constant current of 3 mA/gel. Duplicate gels were run for each protein load. One gel was stained for protein by immersing for 30 min in Coomassie Brilliant Blue G-250 (0.25% in methanol/acetic acid/water: 45/45/10, v/v). After mixing is methanol/acetic acid/water (2/3/35, v/v) for 15 min, gels were then destained electrophoretically for 45 min at 24 V. The other gel was frozen in solid CO_2 and sliced into 1-mm thick discs which were extracted overnight with 1 ml 10 mM sodium phosphate (pH 8)/0.1 M NaCl/ 0.1% Triton X-100 and the activity determined.

Electrophoresis was also performed in the presence of SDS in 5% gels [13]. The results were identical whether proteins were applied altogether on one gel or individually on separate gels. Standards employed were β-galactosidase, phosphorylase *a*, bovine serum albumin, catalase, ovalbumin, and lactate dehydrogenase. Proteins at a concentration of 1 mg/ml were incubated for 5 min at 100°C in 1% β-mercaptoethanol/1% SDS. Staining and destaining were as above.

Sedimentation equilibrium. Molecular weight was determined in the Spinco Model E ultracentrifuge using the meniscus depletion technique of Yphantis [14]. Studies were carried out at 20°C employing interference optics and 7 mm solution columns. The method for decreasing the time to equilibrium was as previously described [15]. Acetylcholinesterase was employed at an intial concentration of 0.5 mg/ml in 10 mM sodium phosphate (pH 7.5)/0.1 M NaCl/5 mM $MgCl_2$/3 mM NaN_3. A partial specific volume of 0.73 ml/g [16] for acetylcholinesterase was used in the calculations. Buffer densities were measured at 20°C in a pycnometer.

Circular dichroism. Spectra were obtained at 25°C with a Cary model 60. The absorbance at 280 nm of acetylcholinesterase solutions was approximately 0.2. Cuvettes of 1-cm pathlength were employed. Calculations of structural parameters were made following the guidelines of Chen et al. [17].

Results and Discussion

The *N*-methylacridinium ligand employed was synthesized by a modification of the Dudai and Silman method [1]. Several investigators have encountered difficulty in the preparation of Compound IV (ref. 18 and unpublished individual observations by Himel, C., Millar, D.B. and Kurlansik, L.). Initially, following the directions of Dudai and Silman [1], we obtained an almost quantitative yield of 9,10-dihydro-9-oxoacridine (acridone). The conversion of 9-aminoalkylacridines to acridone upon exposure to water, base, and heat has been reported [19,20]. Since commercially available 1,2-diaminopropane contains water as the hemi-hydrate, it seemed reasonable that either anhydrous amine or another synthetic method which tolerated water should be employed to prepare IV. The results of our attempts have led to the modifications detailed in Experimental. The formation of acridone has been reduced by employing reaction temperatures of 70°C and below. Furthermore, by reversing the addition of the reactants to provide a more direct synthetic route, formation of the 9-phenoxyacridine intermediate described by Albert [20] is avoided. The preparation of IV as described above is quite reproducible and can be performed by one who has had little prior knowledge of or training in synthetic organic chemistry.

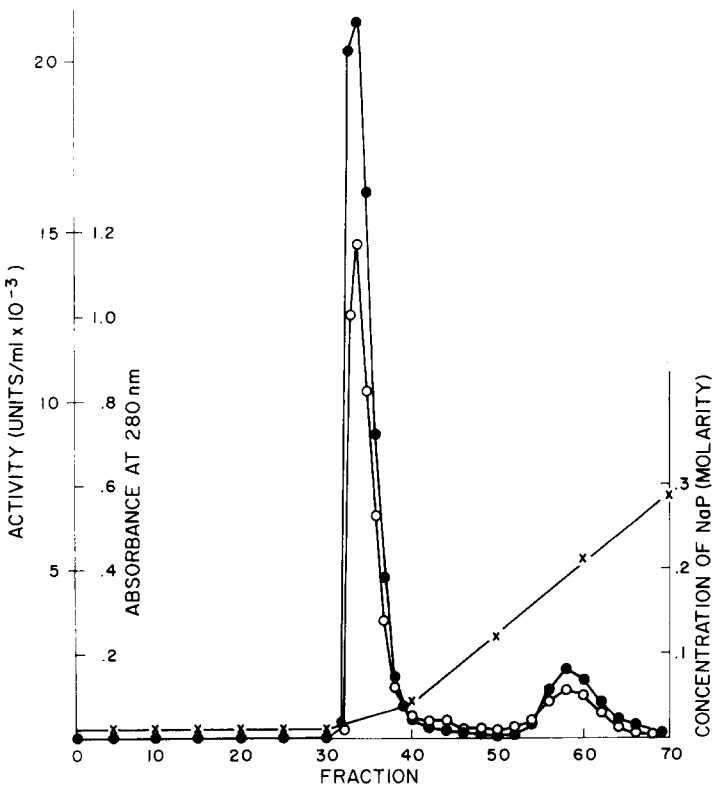

Fig. 1. Hydroxyapatite chromatography of a sample of affinity-purified acetylcholinesterase. Details of chromatographic conditions are given in Experimental. Fractions were assayed for activity (●), A_{280} (○), and phosphate concentration (X).

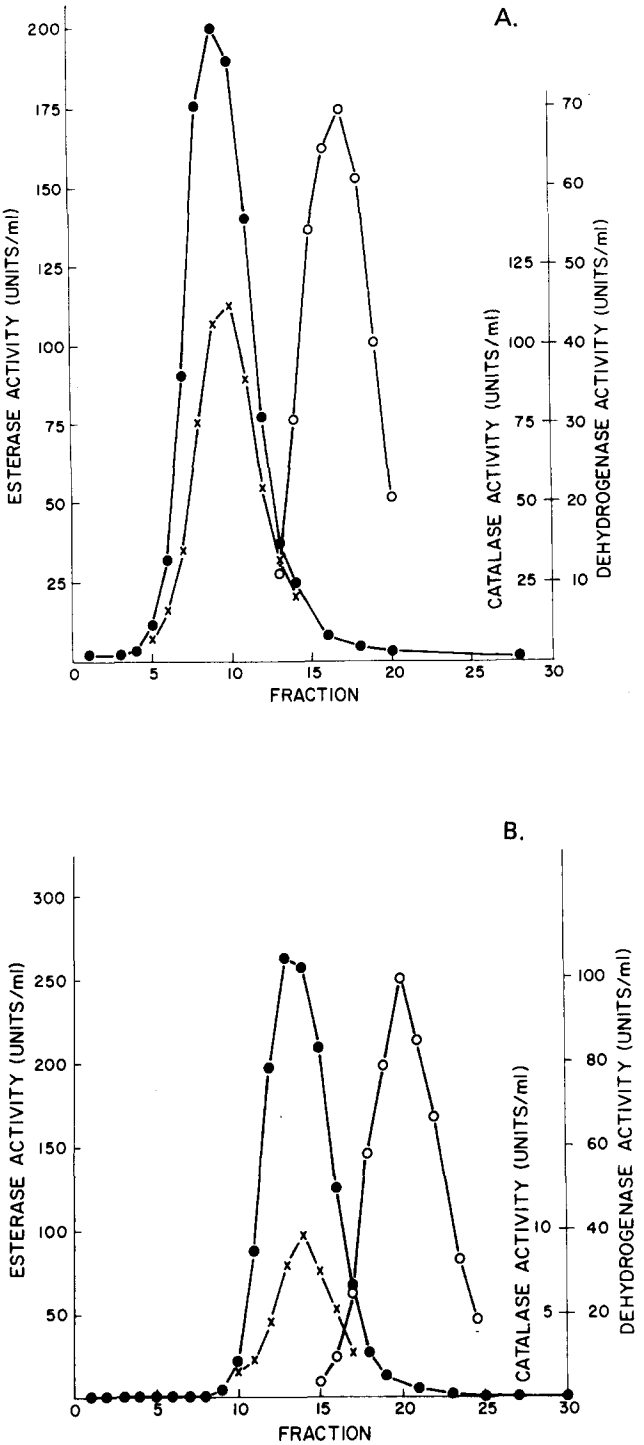

Fig. 2. Sucrose density gradient centrifugation of Peak I isolated after hydroxyapatite chromatography. Conditions are described in Experimental: A, gradients in 0.1 M NaCl; B, gradients in 1.0 M NaCl. Fractions were assayed for acetylcholinesterase (●), catalase (X), and lactate dehydrogenase (○).

Acetylcholinesterase was isolated by affinity chromatography on a column prepared with *N*-methylacridinium. At a ligand concentration of 0.8 μmol/ml gel, a volume of homogenate equivalent to approx. 12 mg acetylcholinesterase can be applied with greater than 95% retention of activity. Enzyme is consistently recovered in greater than 70% yields. Analysis of the sample at this stage of purification by sucrose density gradient centrifugation and polyacrylamide gel electrophoresis indicates that 11-S is contaminated with the higher molecular weight forms.

Some preliminary work in this laboratory has suggested that hydroxyapatite might be an excellent medium for separating the low and high molecular weight forms of acetylcholinesterase. Accordingly, the affinity-isolated enzyme was chromatographed on Bio-Gel HT (Fig. 1). The applied enzyme was resolved into two well separated peaks of activity. The first peak (Peak I) was very sharp and eluted at a sodium phosphate concentration of 0.05 M whereas the second (Peak II) eluted at 0.20 M sodium phosphate and was somewhat broad. This result was also obtained when a crude homogenate was treated with 50% more trypsin. Thus, the presence of Peak II does not appear to be the result of incomplete trypsin digestion. Peak I consistently comprised 85% of the recovered activity and Peak II, 15%. Sucrose density gradient analysis of each peak in gradients of low and high ionic strength is shown in Figs. 2 and 3. Peak I exhibited only one enzymatically active component with an $S_{20,w}$ value of 11.3 irrespective of ionic strength. However, Peak II was comprised of three active components, two of which were the 14- and 18-S forms. The third component exhibited variable behavior, i.e., it sedimented with an $s_{20,w}$ value of 11.3 in low ionic strength and of 9.5 in high ionic strength. We estimated that 14- and 18-S comprised 8% of the recovered activity and "9.5-S", 7%. Polyacrylamide gel electrophoresis confirmed the results above and indicated that the purified 11-S acetylcholinesterase (Peak I) migrated as a single band of protein and activity. However, Peak II had two bands, and each stained for protein and activity. One of the bands corresponded to material that did not enter the gel, presumably 14- and 18-S forms, whereas the other band entered the gel and exhibited an R_F identical to 11-S. The above experimental behavior of the "9.5-S" form emphasizes the point that it would be very difficult to detect in a sample of acetylcholinesterase isolated from trypsin-digested homogenates unless the sample were subjected to chromatography on hydroxyapatite.

Analytical data for the 11-S acetylcholinesterase (Peak I) isolated in our study compared reasonably well to literature values for other preparations. Sedimentation equilibrium showed it to be monodisperse with a molecular weight of approx. 325 000 (Fig. 4). This is in agreement with the value of 323 000 reported by Dudai et al. [21]. Polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol revealed a major component of 59 500 daltons. In addition, minor components were observed with molecular weights of 88 700 and 32 100. These results are qualitatively consistent with those in the literature which attribute this apparent heterogeneity to proteolytic action [2,5,7]. Since other studies may have been done with 11-S that was contaminated with 9.5-S, our SDS results also suggest that these two forms exhibit similar qualitative patterns. Our analysis of the CD spectrum showed that the

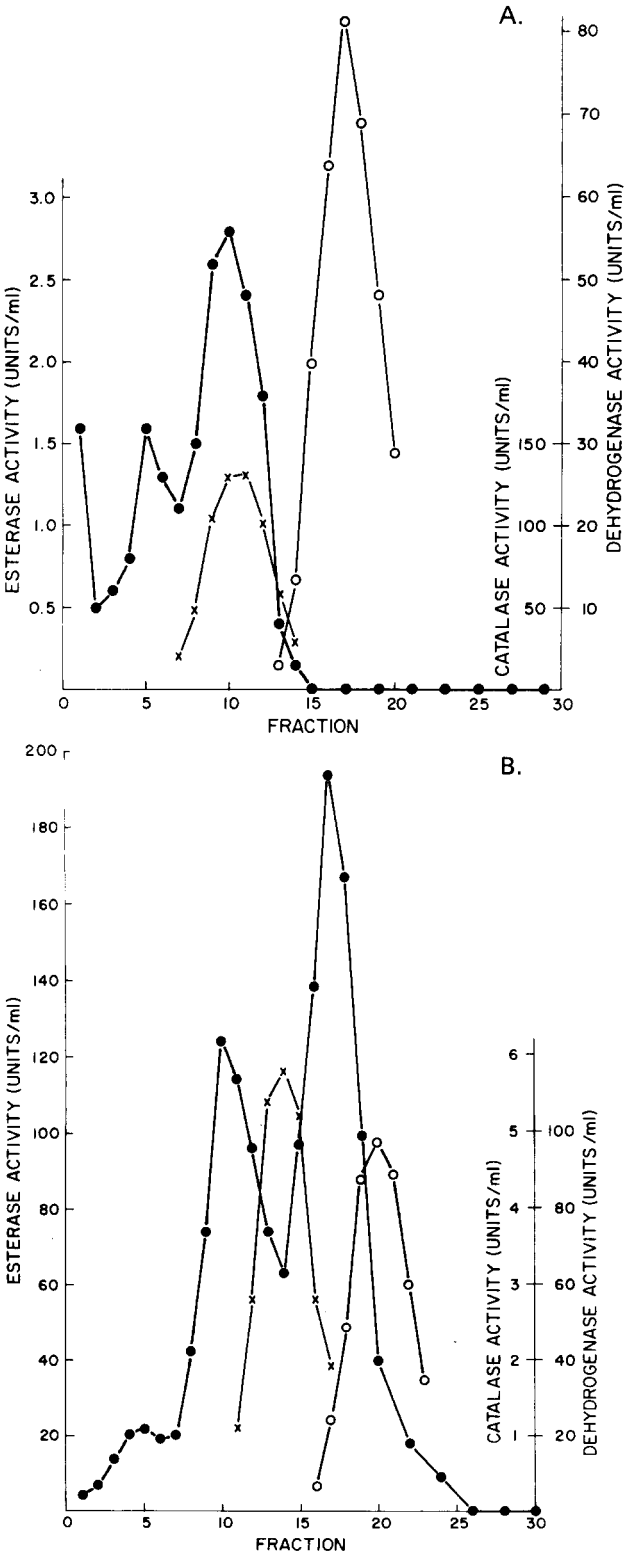

Fig. 3. Sucrose density gradient centrifugation of Peak II isolated after hydroxyapatite chromatography. Conditions are described in Experimental. A. gradients in 0.1 M NaCl; B. gradients in 1.0 M NaCl. Fractions were assayed for acetylcholinesterase (●), catalase (X), and lactate dehydrogenase (○).

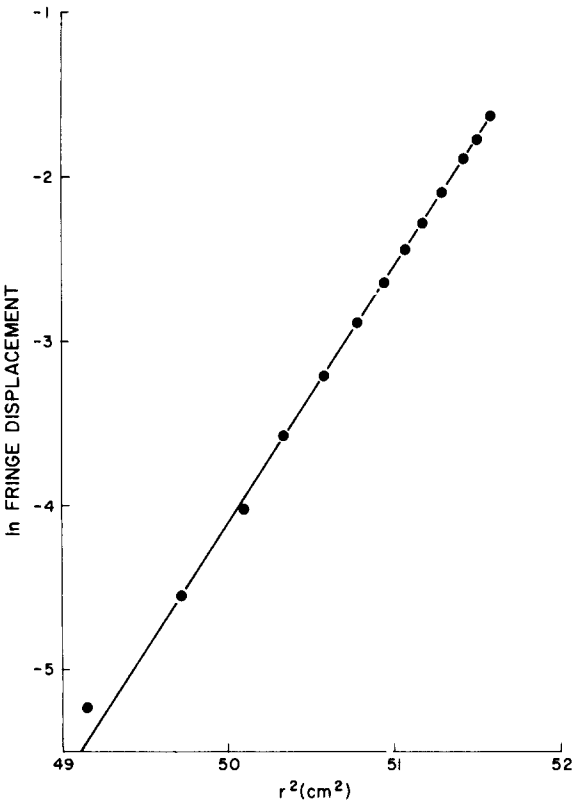

Fig. 4. Sedimentation equilibrium of 11-S acetylcholinesterase at 20°C and a rotor speed of 8000 rev./min. The solvent is 10 mM sodium phosphate (pH 7.5) in 0.1 M NaCl, 5 mM $MgCl_2$ and 3 mM NaN_3.

enzyme exhibited 32% α-helix, 15% β-sheet, and 53% random coil. These data are not in agreement with a value of >90% α-helix obtained by Kitz and Kremzner from ORD measurements [22]. The source of this difference is currently under investigation.

In sum, the results of this study on the homogeneity of 11-S acetylcholinesterase offer a scheme for isolating enzyme that is not detectably contaminated with other active forms of the enzyme, especially the 9.5-S form. Since this form co-sediments with 11-S in 0.1 M NaCl gradients and coelectrophoresis on 6% gels, other procedures that do not employ hydroxyapatite chromatography as a final step most likely result in enzyme that contains 5—10% of the 9.5-S form. Thus, the purification of 11-S acetylcholinesterase as described affords enzyme which may be more suitable for some physicochemical characterizations.

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