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AFFINITY CHROMATOGRAPHY OF ACETYLCHOLINESTERASE FROM *ELECTROPHORUS ELECTRICUS* ELECTROPLAX

INVESTIGATIONS ON 9-AMINOALKYLACRIDINE AFFINITY LIGANDS

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SUMMARY

The neural enzyme 11-S acetylcholinesterase (E.C. 3.1.1.7) was purified by affinity chromatography from a trypsin digest of *Electrophorus electricus* electric organ. Unquaternized affinity ligands were reported which were comparable in efficacy to the routinely employed "quaternized acridine MAC ligand". A study was made of the quaternization reactions of various 9-aminoalkylacridines and 9-aminoacridine along with their relative binding affinities to acetylcholinesterase. Ease of synthesis in conjunction with the column performance of these unquaternized 9-aminoalkylacridine compounds made them the preferred affinity ligand in acetylcholinesterase chromatography. A new carbodiimide synthetic route for these unquaternized ligands was described.

INTRODUCTION

Affinity chromatography utilizing various methyl "quaternized" heterocycles of 9-aminoalkylacridinium ligands has been the preferred isolation method for purification of the neutral enzyme acetylcholinesterase, E.C. 3.1.1.7, from crude extracts of *Electrophorus electricus* electric organ tissue[1–7]. Such columns employ affinity ligands that bind with enzyme as a non-covalent, equilibrium complex. The preferred ligand, reportedly a 10-methylacridinium derivative (MAC), was attached to the insoluble support matrix through alkyl chain spacer arms of varying lengths. These "MAC" columns have been used in the isolation of both high (14-S and 18-S) and low (11-S) molecular weight forms of acetylcholinesterase with the 11-S form appearing to predominate in tryptic digests of crude eel extracts[7].

The "MAC" ligand (Fig. 1; compound VII) was first synthesized by Dudai and Silman[2]. The primary synthetic difficulty reported in its preparation was unwanted conversion of the 9-aminoalkylacridine to 9-oxoacridine (acridone) by hydrolytic reactions. In addition, the actual identity of the quaternary methyl moiety of the "MAC" ligand (as opposed to a hydrohalide salt) was never proven unequivocally by critical spectral investigation, *i.e.*, nuclear magnetic resonance (NMR) (a means best

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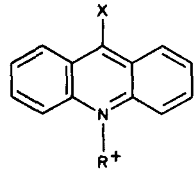

I: R = No substituent; X = $NH\text{–}CH_2\text{–}CH(CH_3)\text{–}NH_2$

IIA: R = No substituent; X = $NH\text{–}CH_2\text{–}CH(CH_3)\text{–}NH\text{–}C(=O)\text{–}(CH_2)_5\text{–}NH_2 \cdot 2\,HBr$

IIB: R = No substituent; X = $NH\text{–}CH_2\text{–}CH(CH_3)\text{–}NH\text{–}C(=O)\text{–}(CH_2)_5\text{–}NH\text{–}C(=O)O\text{–}CH_2\text{–}C_6H_5 \cdot I^-$

IIC: R = No substituent; X = $NH\text{–}CH_2\text{–}CH(CH_3)\text{–}NH\text{–}C(=O)\text{–}(CH_2)_5\text{–}NH_3^+\ Br^-, Br^-$

III: R = No substituent; X = NH_2

IV: R = CH_3; X = NH_2

V: R = CH_3; X = No substituent

VI: R = No substituent; X = H

VII: R = CH_3; X = $NH\text{–}CH_2\text{–}CH(CH_3)\text{–}NH\text{–}C(=O)\text{–}(CH_2)_5\text{–}NH_3^+\ Br^-$

Fig. 1. Chemical structure of various acridine ligands.

suited for its detection). Through such spectra we have discovered that the 9-aminoalkylacridine heterocycle is difficult to quaternize without commensurate degradation of the 9-aminoalkyl moiety[6].

These considerations prompted an investigation of other 9-aminoalkyl affinity ligands within the acridine series. Our results indicated that the unquaternized analogue of the "MAC" ligand can be prepared and coupled to a Sepharose gel by a simpler two step carbodiimide route. This unquaternized analogue provides an affinity column commensurate in efficiency to previously described "MAC" columns.

EXPERIMENTAL

Materials

Electric eels (*E. electricus*) were purchased from Paramount Research Supply Co., Sepharose 4B was obtained from Pharmacia, and hydroxyapatite (Bio-Gel HT) from Bio-Rad Labs. Acetylthiocholine iodide, 5,5′-dithiobis(2-nitrobenzoic acid) and bovine serum albumin were obtained from Sigma; CNBr, 1,2-diaminopropane, 1,2-diaminoethane, 6-aminocaproic acid, acridine, N-benzyloxycarbonyl-ε-aminocaproic acid and methyl iodide from Aldrich. The 9-chloroacridine was supplied by Eastman and decamethonium bromide by Pfaltz and Bauer. Trypsin and soy bean trypsin inhibitor were from Worthington.

Estimation of protein concentration and enzyme activity

Protein was estimated by tryptophane absorption at 280 nm using the value of $A_{280} = 18$ (ref. 8). Enzyme activity was measured by the colorimetric procedure of Ellman *et al.*[9], where an activity of one unit was equivalent to one A_{412} unit per min. Ellman related absorption assays and spectra were recorded on a Beckman Model 25 double beam spectrophotometer equipped with a temperature-controlled cell holder at 20°C.

Synthesis of "MAC" affinity ligand

The synthesis of the "MAC" affinity ligand of Dudai and Silman (10-methyl-9-[N^{β}-(ε-aminocaproyl)-β-aminopropylamino]acridinium bromide), compound VII, was attempted using the modified preparatory scheme of Christopher *et al.*[1]. Due to numerous problems encountered by ourselves and others, a detailed NMR survey of the major precursors (compounds I and IIB) of the "MAC" ligand was undertaken. Proton NMR spectra were obtained on a Varian Model HA-100 utilizing tetramethylsilane as an internal chemical-shift standard. Detailed spectral data, exceeding that presented here, are available upon request.

Synthesis of acridine derivatives

The quaternary salts of acridine and 9-aminoacridine were prepared by reaction with an excess of methyl iodide in a sealed bottle at 40°C. Approximately quantitative yields were obtained after several days. The products precipitated from the reaction solution and were recrystallized from methanol. Further purification was accomplished by Soxhlet extraction employing anhydrous diethyl ether. This removed any coprecipitated acridine starting material. Compound V melted at 228–230°C and compound IV > 300°C.

Compound IIA was prepared from I by reaction with N-benzyloxycarbonyl-ε-aminocaproic acid according to the method of Dudai and Silman[2]. The 9-(N^{β}-[N-benzyloxycarbonyl-ε-aminocaproyl]-β-aminopropylamino)acridine intermediate was isolated and the benzyloxycarbonyl protecting group was removed by reaction with anhydrous HBr in glacial acetic acid.

Synthesis of unquaternized acridine affinity gel (Fig. 2)

The "MAC" analogue ligand, compound IIA, was attached to the gel matrix using a modified carbodiimide coupling procedure first employed by Ralston *et al.*[10] in a procainamide affinity column for butyrylcholinesterase (E.C. 3.1.1.8).

Sepharose 4B was activated at 4°C by adding 175 ml of 5 *M* phosphate buffer (3.35 mol K_2HPO_4 and 1.67 mol K_3PO_4 per liter; pH 12.2) to 200 ml of settled gel beads. To this stirred gel suspension a 70 ml CNBr in water solution (40 mg, 377 μmol, per ml settled gel) was added over a 2 min period. A total reaction time of 10 min, including addition, was allowed. The activated gel suspension was rapidly filtered and washed with 1 l of ice cold 0.25 *M* $NaHCO_3$ buffer (pH 9). Coupling of 6-aminocaproic acid was accomplished by immediately adding the cold gel to 200 ml of cold 0.25 *M* $NaHCO_3$ buffer (pH 9) containing 6-aminocaproic acid (13 mg, 100 μmol, per ml of gel). The reaction was complete at 4°C after 18 h. The gel was then thoroughly washed with distilled water and stored at 4°C. Fluram analysis for primary amines present in the gel washings showed the coupling reaction to be quantitative[11].

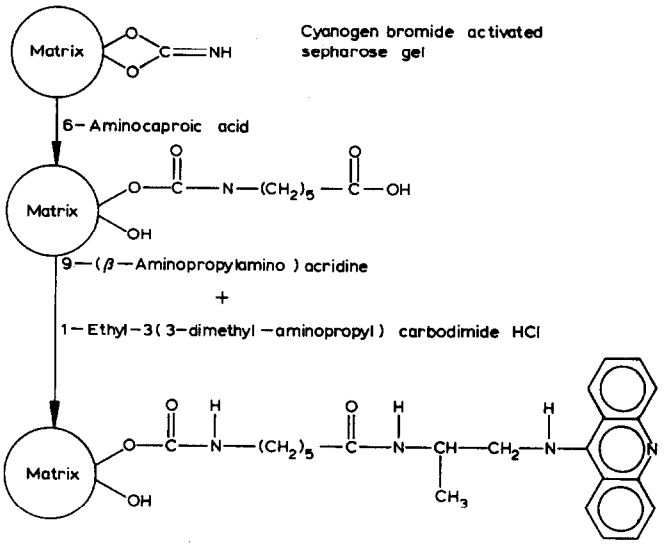

Fig. 2. Synthesis of unquaternized acridine affinity ligand by carbodiimide method.

An aqueous solution of I was prepared by suspending the powder (0.389 mg, 1.56 μmol, per ml gel) in 100 ml of water and titrating with 1 *M* hydrochloric acid until dissolution occurs. The resulting solution was added to a stirred gel suspension containing 200 ml of settled 6-aminocaproic-coupled gel in 100 ml of water. The pH of the suspension was adjusted to 4 using 1 *M* sodium hydroxide solution. Then 4 g of solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added slowly. After 15 h at a pH between 4 and 5 the reaction was complete and the ligand–gel product was washed with distilled water. The absorption of all washings was monitored at 414 nm to measure unbound ligand (λ_{max} = 414 nm, ε = 8500 M^{-1} cm^{-1}). The amount of ligand–gel conjugate was calculated, by difference, to be 1.1 μmol per ml gel. An affinity column (2.5 × 81 cm; 400 ml packed gel) was equilibrated with several volumes of flushing buffer (10 m*M* phosphate, 1 *M* NaCl and 3 m*M* NaN_3 at pH = 7.5).

Enzyme extraction

Acetylcholinesterase was obtained at 4°C from frozen electric eel tissue by homogenizing 200 g of tissue in 300 ml of flushing buffer. The homogenate was incubated with trypsin (10 mg per ml buffer) for 20 h at room temperature. The digestion was halted by addition of soy bean trypsin inhibitor (15 mg per ml buffer) for 30 min. The tryptic digest was centrifuged at 40,000 *g* for 2 h at 4°C. The pellet and lipid layer were removed by filtration through a cotton plug.

Affinity chromatography

Crude acetylcholinesterase (350 ml tryptic digest) was applied to the affinity column at a flow-rate of 5 ml/h. The column was then washed with a minimum of four volumes of flushing buffer or until $A_{280} < 0.07$. The eluate was collected in 15-ml fractions and monitored for both A_{280} and activity (Fig. 3). Enzyme was then dis-

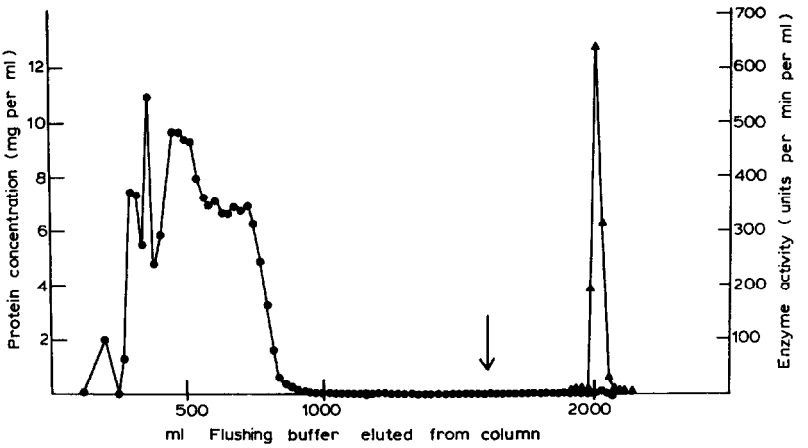

Fig. 3. Affinity chromatography of trypsin digested *E. electricus* homogenate employing the unquaternized 9-alkylaminoacridine affinity ligand. Details of chromatographic conditions are given in Experimental section. Fractions were assayed for enzymatic activity (▲) and for protein concentration (●). The arrow indicates the start of decamethonium bromide elution to release enzyme.

placed from the affinity ligand by eluting with 20 m*M* decamethonium bromide in flushing buffer. Active fractions were pooled and concentrated at 4°C under 20 lb./in.^{2} nitrogen employing an ultrafiltration pressure cell with a Pellicon Type PT membrane. The concentrate (approximately 10 ml) was first dialyzed against three changes of flushing buffer to remove decamethonium ions, then dialyzed against three changes of 10 m*M* phosphate, 0.1 *M* NaCl, 10 m*M* $MgCl_2$, 3 m*M* NaN_3 buffer (pH 7.5) in preparation for hydroxyapatite chromatography.

Hydroxyapatite chromatography

The purification of the 11-S molecular form of acetylcholinesterase followed the hydroxyapatite chromatography procedure of Christopher *et al.*[1].

Binding affinity of ligands

Enzyme-inhibitor systems were investigated by the Ellman assay for enzyme activity. Most inhibitors of acetylcholinesterase complex reversibly with the enzyme in a mixed competitive–uncompetitive fashion (with deacylation rate limiting substrates; *i.e.*, acetylcholine and acetylthiocholine)[12]. For our purposes a simple analysis of only the competitive component, representative of ligand binding to the anionic subsite of the catalytic site, was adequate. The data were analyzed using the value for K_{D1}, the dissociation constant for the inhibitory ligand at the active site.

All measurements were made at 20°C on a Beckman Model 25 spectrophotometer, equipped with recorder and thermostated cell compartment. Analysis of data were similar to that of Balcom and Fitch[13]. During the initial phase of the progress curve, the absorbance corresponds to the percentage of substrate hydrolyzed, with the slope corresponding to the reaction velocity.

Purified 11-S acetylcholinesterase was diluted 400 times from a 0.5 mg/ml stock solution into 0.05 *M* phosphate buffer (ionic strength 0.113 and pH 7) containing 3 m*M* NaN_3 and 0.5 mg/ml bovine serum albumin. Lineweaver–Burk plots yielded

straight lines for the following substrate concentrations: 0.04, 0.05, 0.06, 0.08, 0.10, 0.12 and 0.15 m*M*. The velocity expression

$$\frac{1}{v} = \frac{\frac{k_{-1} + k_2}{k_1}\left(1 + \frac{[I]}{K_{D1}}\right)}{v_m} \cdot \frac{1}{[S]} + y\text{-intercept} \qquad (1)$$

was derived in slope–intercept form suitable for Lineweaver–Burk graphical analysis, where relative velocity, v, was a function of inhibitor ligand (I) and substrate (S) concentration, the velocity maximum, v_m, the Michaelis addition complex equilibria, k_1 and k_{-1}, and the acylation rate, k_2. The dissociation constant, K_{D1}, of the ligand at the active site was obtained from a secondary replot of Lineweaver–Burk slope *vs.* ligand concentration. Division of the secondary plot y-intercept by its slope yields K_{D1}. All values for slopes and y-intercepts were obtained from least squares regression analysis.

RESULTS AND DISCUSSION

The 9-aminoalkylacridine affinity ligands, utilized extensively in acetylcholinesterase preparative affinity columns, bind primarily to the anionic subsite of the active site. Upon binding, a two point involvement of the ligand with the anionic subsite's protein surface occurs[14]. The first being the coulombic interaction of the ligand's charged heterocyclic nitrogen with the anionic center. The second is the hydrophobic interaction of the polyacene ring system with the cleft-like protein surface of the anionic subsite. The planar conformation of the acridine ring permits a maximal binding configuration within the "jaws" of this cleft[15], resulting in low dissociation constants (10^{-7} and 10^{-8} *M*) for acridine derivatives. These high binding affinities have made the 9-aminoalkylacridine ligands useful in the affinity purification of acetylcholinesterase.

Several investigators, including ourselves, have encountered difficulty in the preparation of the "MAC" affinity ligand of Dudai and Silman. The conversion of compound I to acridone upon exposure to water, base and heat was successfully eliminated by the improved synthetic method of Christopher *et al.*[1]. In our laboratory, following the instructions of Dudai and Silman, we were unable to prepare the quaternary intermediate (methyl quaternary analogue of compound IIB) or the "MAC" ligand itself (compound VII).

Dudai noted the possibility of two possible isomeric products in the preparation of I, corresponding to the methyl group on the alpha and beta carbons; however, no attempt was made by them to quantitate amounts of each isomer present. Integrations from NMR spectra of I dissolved in [^{2}H]chloroform–[$^2H_2$]water (90:10) indicated an isomer composition of 70% beta and 30% alpha, based on methyl group signals (beta isomer doublet at 2.19 ppm, alpha isomer doublet at 2.50 ppm). The position of the methyl group does not appear to have any effect on the subsequent reactions involved in ligand synthesis. Substitution of 1,2-diaminoethane for 1,2-diaminopropane in ligand synthesis yields a compound analogous to I; however, it is more susceptible to di-substitution by 9-chloroacridine in the inital reaction. This

product is also more reactive to acridone formation. Attempts to isolate the 9-(2-aminoethylamino)acridine analogue as the free base yields predominantly acridone. This analogue could be isolated only as the hydrochloride salt, thereby indicating that steric factors involving the methyl group of compound I are possibly responsible for the reduced activity of the primary amine to nucleophilic displacement reactions.

NMR analysis of the product obtained from the methyl iodide quaternization reaction of "MAC" indicates that, in our hands, the product is the hydroiodide salt of the starting material rather than the reported acridinium compound. Spectra of methyl quaternary analogues of "MAC" obtained in [$^2H_6$]dimethyl sulfoxide displayed a sharp singlet peak at 5.0 ppm (compound V) and 4.2 ppm (compound IV) for their quaternary methyl moieties. A corresponding singlet of similar chemical shift could not be found in the product (compound IIB) of the "MAC" methyl iodide reaction. Similar reactions in other solvent systems (acetone, acetonitrile, dimethyl sulfoxide and isopropanol) yielded starting material and/or the hydroiodide salt.

A possible explanation for the preferential hydroiodide salt formation over quaternization of the acridine heterocycle may be found in the nature of the solvent employed and the basicity of the acridine heterocycle. In the presence of a base catalyst, such as a 9-aminoalkylacridine (pK_a 9–10), the alcoholic solvent could undergo a Williamson addition with the alkyl halide yielding an asymmetric alkyl ether and hydrogen iodide[16]. This results in the formation of a hydroiodide salt at the acridine heterocycle. The Williamson reaction becomes a significant side reaction in alcoholic solvents when quaternization proceeds slowly, which appears to be the case with 9-aminoalkylacridines. Attempts to employ more vigorous reaction conditions, *i.e.*, increased temperature, or other more reactive methylating agents, *i.e.*, methyl fluorosulfonate, resulted in the degradation of the ligand's alkyl chain.

Our failure to synthesize quanternary affinity ligands prompted us to search for other viable alternatives within the acridine series. Previous studies in this laboratory indicated that quaternization of the heterocycle of 9-aminoacridine did not increase binding affinity at the anionic subsite of acetylcholinesterase[15]. The omission of the quaternization step would greatly facilitate ligand synthesis by permitting the use of a facile carbodiimide route for the amide spacer moiety.

Under typical conditions, dissociation constants for affinity ligands at the active site of acetylcholinesterase are obtained in a low ionic strength (<0.12) environment. This is done in order to decrease competitive binding interference from positive ions of the buffer medium. However, on acridine affinity columns the ligand–enzyme binding is in the presence of 1 *M* NaCl (flushing buffer). This high salt concentration prevents non-specific protein retention via the ion-exchange groups of the Sepharose support medium. This high ionic strength results in a reduction of the ligand affinity for enzyme. In order to obtain a representative binding profile, dissociation constants (Table I) for potential column affinity ligands are measured in both flushing and low ionic strength (0.113) phosphate buffer. The former being the more accurate representation of binding affinity under preparative column conditions.

In general, the acridine ligands investigated displayed a marked reduction of binding affinity in flushing buffer when compared to values obtained in low ionic strength buffer. Table I shows binding dramatically increasing from quaternization (compound V) of the heterocycle of acridine (compound VI). This is due to the increased positive charge associated with the quanternary nitrogen of V; however, the

TABLE I

DISSOCIATION OF VARIOUS ACRIDINE AFFINITY LIGANDS

No.	*Compound*	*Catalytic site dissociation constant ($\times 10^{-7}$ M)*	
		Flushing buffer	*Low ionic strength buffer*
I	9-(β-Aminopropylamino)-acridine	4.88 ± 0.981	1.25 ± 0.238
IIA	9-[N^{β}-(ε-Aminocaproyl)-β-aminopropylamino]acridine dihydrobromide	8.04 ± 0.512	2.43 ± 0.341
IIB*	9-[N^{β}-(N-Benzyl-oxycarbonyl-ε-aminocaproyl)-β-aminopropylamino]-acridine iodide	3.95 ± 0.319	1.83 ± 0.211
IIC**	9-[N^{β}-(ε-Amino-caproyl)-β-aminopropyl-amino)acridine bromide hydrobromide	7.54 ± 0.891	2.54 ± 0.423
III	9-Aminoacridine	0.480 ± 0.0132	0.258 ± 0.0210
IV	10-Methyl-9-amino-acridinium iodide	4.74 ± 0.612	1.20 ± 0.300
V	10-Methylacridinium iodide	5.03 ± 0.512	1.44 ± 0.220
VI	Acridine	126.0 ± 4.23	40.0 ± 1.23
VIII	2-Methhoxy-6-chloro-9-aminoacridine	1.42 ± 0.0932	0.652 ± 0.0124

* Intermediate product (Christopher's modifications of Dudai and Silman's directions).
** "MAC" ligand product (Christopher's modifications of Dudai and Silman's directions).

electronic nature of the heterocycle in 9-aminoalkylacridines are substantially different than acridine. The amino group at the 9-position of compounds I–III is a powerful electron withdrawing moiety which causes delocalization of the non-bonding electron pair of the 9-amino group into the aromatic system. A net positive charge is thereby present on the nitrogen heterocycle in aqueous solutions (pH < 8) without the necessity of quaternization[17]. Hence, the binding of the N-methyl quaternary analogue of 9-aminoacridine (compound IV) is markedly reduced from that of 9-aminoacridine. The reduced affinity is a consequence of the addition of a bulky methyl group onto the heterocycle. This methyl group positions itself, on binding, between the two charged moieties on the enzyme and ligand. Steric hindrance prevents a maximal binding configuration, *i.e.*, the charge on the ligand is pushed away from the anionic center of the enzyme. This methyl group's steric effect decreased catalytic site binding by approximately an order of magnitude[15]. Smaller binding differences, displayed by the other compounds in the series, are generally caused by either interference or association of the ligands 9-aminoalkyl chain with the protein surface of the enzyme.

The binding study compiled in Table I led us to believe that the unquaternized analogue of Dudai and Silman's "MAC" ligand would perform suitably on an affinity column. Acetylcholinesterase was isolated by affinity chromatography on a 400-

ml gel column prepared with the unquaternized acridine ligand. At a ligand concentration of 1.1 μmol per ml gel, a volume of homogenate representing approximately 8 mg of acetylcholinesterase could be introduced with a 93–95% retention of activity. Enzyme was recovered in 80–90% yield based on activity of the decamethonium bromide elution fractions (specific activity 8930 μmol/min · mg). This efficacy was commensurate with affinity columns employing supposed quaternary acridinium ligands[1,2,7]. As seen in Fig. 3, the majority of ancillary protein was eluted within two to three column volumes of flushing buffer. Elution with this buffer was continued for several more column volumes after the major protein peak to insure that all non-enzymatic protein was washed from the gel.

In general, we found that between 0.75 and 1.1 μmol per ml gel was the optimum ligand concentration. A lower concentration caused insufficient enzyme retention; whereas, a greater concentration resulted in a 100% retention of enzyme activity but recovery of enzyme with decamethonium bromide elution was very poor (< 50%).

Pure 11-S acetylcholinesterase (molecular weight 320,000) was obtained from the affinity column elutate by hydroxyapatite chromatography. The applied enzyme was eluted in two well defined peaks of activity. The 11-S fraction was eluted in a sharp peak at a sodium phosphate concentration of approximately 0.06 *M*. The second peak was comprised of three active forms, 9.5-S, 14-S and 18-S, and was eluted as a very broad peak at 0.20 *M* sodium phosphate. This was in accordance with previous research[1].

In summary, there appears to be considerable doubt, based on NMR spectral evidence, as to whether previously employed "MAC" affinity ligands are indeed quaternary acridinium compounds but rather hydroiodide salts. This controversy becomes academic in light of the fact that unquaternized 9-aminoalkylacridine ligands function elegantly in the affinity chromatography of acetylcholinesterase. In addition, the speed and ease of the synthesis of these unquaternized compounds makes them the ligands of choice in acetylcholinesterase purification. Other readily available acridine derivatives, such as 2-methoxy-6-chloro-9-aminoacridine (compound VIII), were investigated for their utility in affinity chromatography; however, none appears to possess the necessary binding affinity shown by the 9-aminoalkylacridine ligands.

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