271. Affinity Chromatography with ε-Aminocaproyl-choline Derivatives[1])

(Preliminary Communication)

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Zusammenfassung. An Sepharose 2B gebundene Derivate von ε-Aminocaproylcholin vermögen Acetylcholinesterase und andere, vermutlich ebenfalls für Acetylcholin spezifische Proteine aus Elektroplax von *Electrophorus electricus* bevorzugt zu adsorbieren. Die Elution erfolgt mit Tetramethylammonium-chlorid.

Introduction. – In order to be able to compare chemical features of the recognition sites of acetyl cholinesterase (AChE) and of acetyl choline receptor molecules (AChR) for acetyl choline (ACh), we have prepared diazoacetyl choline bromide (DACh) [1]. This compound proved to be a potent ACh agonist and a competitive inhibitor of AChE ($K_i \approx 10^{-4}$). It is hydrolyzed only very slowly by AChE; irradiation of the mixture of DACh and AChE at $\lambda \approx 360$ nm causes irreversible inhibition of AChE by covalent attachment of (^{14}C-labelled) DACh [2]. Photolysis also produces irreversible reaction with motor endplates [3].

The next stage of our work requires highly purified AChE and AChR. For the purpose of isolation and, possibly, of separation of the two, we are studying the use of affinity chromatography [4].

AChE has been purified on columns of agarose (sepharose) containing a covalently attached *enzyme inhibitor*, *p*-aminophenyl-trimethylammonium chloride (PTA; $K_i \approx 10^{-6}$ M) [5]–[7]. Elution was performed with NaCl gradients or strong, competitive AChE inhibitors like tensilon (*m*-hydroxyphenyl-dimethyl-ethylammonium chloride) or decamethonium. Using a PTA sepharose, **2**, according to *Berman* & *Young* [5], we found that *tetramethyl-ammonium chloride* offers certain advantages as eluant. In particular, assays of enzyme activity may be performed without previous dialysis of the samples, as is necessary with the strong inhibitors mentioned above.

Using a potent ACh antagonist, cobrotoxin bound to Sepharose 4B, *Changeux* & *Boquet* [8] have succeeded in very strongly adsorbing AChR. In the case of such strong and effective interactions, it is, however, very difficult to desorb and retrieve the desired protein.

In order to be able to retain and recover macromolecules that are capable of specifically 'recognizing' ACh, we are investigating the capabilities of *agonists* and *weak inhibitors* more closely related to ACh to serve as ligands in affinity chromatography. This paper gives a preliminary account of the use of ε-aminocaproyl choline (Aca-choline, **3a**) covalently attached by means of a 'spacer' to Sepharose 2B (affinity adsorbant **3**). The results are very promising.

[1]) Studies on the Chemical Mechanism of Acetyl Choline Action, III. Communication I [1]; II [2]. Part of the thesis of *J. F.*

Materials and Methods. – The following *chemicals* were used: sodium dihydrogen phosphate (p.a. *Merck*), sodium chloride (p.a. *Merck*), magnesium chloride (p.a. *Merck*), tetramethylammonium chloride (purum *Fluka*), ammonium acetate (puriss. *Fluka*), ammonium sulfate (biochem. Zwecke, *Merck*), acetyl choline iodide (puriss. *Fluka*), Sepharose 2B (*Pharmacia*), Diazo-Blue B (*Sigma*), and β-naphthyl acetate (*Sigma*).

AChE activity was measured by the pH-stat method (instrument of *Radiometer*, Kopenhagen) according to *Kremzner & Wilson* [9] by direct addition of eluate aliquots (containing $Me_4N^+Cl^-$, Fig. 1) to a solution of 0.15 M NaCl, 0.04 M $MgCl_2$, 1.7 mM acetyl choline iodide, and 0.1% albumin in N_2 atmosphere at pH 7, 25°. 1 unit (U) of enzyme activity represents 1 μmol of AChI hydrolyzed per minute. Specific activity was calculated from absorbance at 280 nm, using $E_{280}^{1\%} \equiv 18.0$ [7].

Gel electrophoresis was carried out in small, vertical 7.5% polyacrylamide gel columns containing a tris-glycine buffer, pH 8.3 [10], at 20° in a current of 5 mA per gel. Protein was stained with Coomassie Brillant Blue, AChE-activity was detected by the reaction of Diazo Blue B with the β-naphthol released by enzymic hydrolysis of β-naphthyl acetate in 0.05 M phosphate buffer, pH 7 [11].

Crude AChE solution (approx. 1400 U/ml) was prepared from toluene-treated *Electrophorus electricus* electroplax according to *Leuzinger & Baker* [12] (steps 1 and 2) by ammonium sulfate precipitation.

Sepharosyl-oxycarbimino-aminopropyl-(1,3-)aminopropyl-(1,3)-amidosuccinyl-aminopropyl-(1,3)-aminopropyl-(1,3)-aminosuccinic acid, **1**, and *sepharosyl-oxycarbimino-aminopropyl-(1,3)-aminopropyl-(1,3)-amidosuccinyl-aminopropyl-(1,3)-aminopropyl-(1,3)-amidosuccinyl-*p*-aminophenyl-trimethylammonium salt*, **2**, were prepared according to *Berman & Young* [5], using Sepharose 2B and cyanogen bromide (according to *Tesser, Fisch & Schwyzer* [13], the structural link formed by cyanogen bromide is *predominatly* of the isourea type; cf. *Axén* [14]).

Sepharosyl-oxycarbimino-aminopropyl-(1,3)-aminopropyl-(1,3)-amidosuccinyl-aminopropyl-(1,3)-aminopropyl-(1,3)-amidosuccinyl-ε-aminocaproylcholine salt, **3**, was prepared from ε-aminocaproyl-choline chloride, **3a** (synthesis to be described elsewhere [15]), and **1** with a water-soluble carbodiimide in a manner analogous to **2**. 1 ml of packed gel contained between 2 and 5 μmol of ligand.

Proposed reactive chemical structures of affinity adsorbants

(Seph- = sepharosyl, agarosyl; Aca- = ε-aminocaproyl):

1 Seph—O—C(=NH)—[$NHCH_2CH_2CH_2NHCH_2CH_2CH_2NH$—$COCH_2CH_2CO$]$_2$—OH

2 Seph—O—C(=NH)—[$NHCH_2CH_2CH_2NHCH_2CH_2CH_2NH$—$COCH_2CH_2CO$]$_2$—NH—C$_6H_4$—$NMe_3^{\oplus}X^{\ominus}$

3 Seph—O—C(=NH)—[$NHCH_2CH_2CH_2NHCH_2CH_2CH_2NH$—$COCH_2CH_2CO$]$_2$—$NH(CH_2)_5$—CO—$OCH_2CH_2NMe^{\oplus}X^{\ominus}$

3a Aca-choline salt: $H_2N(CH_2)_5CO$—$OCH_2CH_2NMe_3^{\oplus}X^{\ominus}$

Results and Discussion. – The elution profiles (Fig. 1) with PTA (A) and Aca-choline (B, C) ligands are very similar, both with respect to protein absorbance and AChE activity. Two essentially inactive protein peaks emerge at the lower ionic strengths. The second of these two is eluted from PTA sepharose with 0.6 M NaCl, whereas it appears already with 0.2 M NaCl in the case of Aca-choline sepharose. This difference is hard to explain on the basis of ion-exchange effects alone, because both ligands bear essentially the same cationic groups (quaternary ammonium). We intend to explore the possibility of a *specific* interaction between the second peak proteins

and ACh, and of these proteins being potential AChR molecules (for the definition of 'potential receptor molecules' *cf. Schwyzer & Schiller* [16]).

AChE activity appears as one sharp peak from the PTA column after addition of 0.2 M Me_4NCl. It represents 50% of the applied activity; its specific activity is 6500 U/mg. *Berman & Young* [5] report 80% and a maximum of 16.000 U/mg, using 0.1 M NaCl and Tensilon; with Aca-PTA ligands and decamethonium [6] or NaCl gradients [7], yields of 40% and between 35–75%, and specific activities of the most highly purified

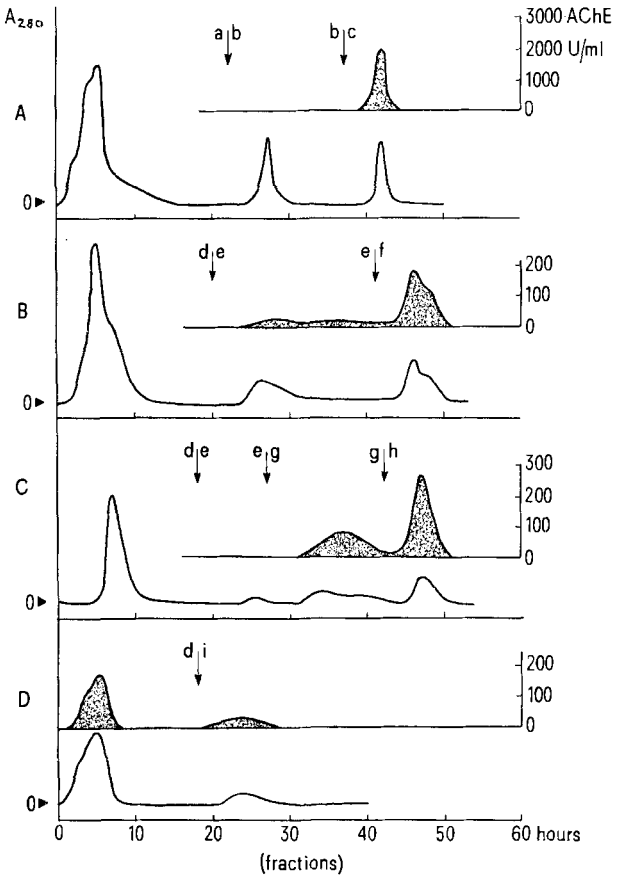

Fig. 1. *Elution profiles of protein* ($A_{280\,nm}$) *and AChE activity* (*U/ml*) *at* +4°

Absorbance in arbitrary units; ○ ▶ = base line. Arrows indicate change of eluant: a) 0.04 M $MgCl_2$ in 0.01 M phosphate buffer, pH 7, 0.2 M NaCl; b) same as a, but 0.6 M NaCl; c) same as a, but 0.6 M NaCl plus 0.2 M Me_4NCl; d) 0.02 M $MgCl_2$ in 0.005 M phosphate buffer, pH 7, 0.1 M NaCl; e) same as d, but 0.2 M NaCl; f) same as d, but 0.6 M NaCl plus 0.2 M Me_4NCl; g) same as d, but 0.3 M NaCl; h) same as d, but 0,7 M NaCl plus 0.2 M Me_4NCl; i) same as d, plus 0.4 M Me_4NCl. **A**: 60 ml (84,000 U) crude AChE on 75 ml PTA-Sepharose, **2**. Flow rate 20 ml/h. **B**: 2 ml (2800 U) crude AChE on 10 ml of Aca-choline-Sepharose, **3**. Flow rate 2 ml/h. Peak activity absorbance, E_{280} = 0.17. **C**: 6 ml (8400 U) crude AChE on 25 ml **3**. Flow rate 6 ml/h. Peak activity absorbance, E_{280} = 0.185. **D**: 1 ml (1400 U) crude AChE on 10 ml spacer-substituted Sepharose, **1**. Flow rate 2 ml/h.

fractions of (recalculated) 9200 and 10,000 U/mg, respectively, have been obtained. Crystalline AChE is active in the range of 12,500 U/mg [12]. We have not attempted to improve yields and specific activities, but have used the PTA adsorbent only to be able to judge the results with our Aca-choline ligand (our lower specific activity is most probably due to the fact that we have assayed the pooled fractions under the active peak, and not a selection thereof).

From the Aca-choline adsorbent (Fig. 1, **B**, **C**), AChE activity is eluted in essentially 2 portions in a total yield of about 65%. A first, very broad area contains about 15%, and a second, rather sharp peak about 50%. The specific activities of the latter peaks were found to be 1900 (B) and 2800 (C) U/mg.

A control run with ligand **1** (Fig. 1, **D**) demonstrated the efficacy of the other ligands in retaining AChE: over 70% of the total activity was eluted with one or two column volumes of 0.1 M NaCl; however, 15% were retained and appeared at higher ionic strength. We haven't investigated the reason for this partial retention.

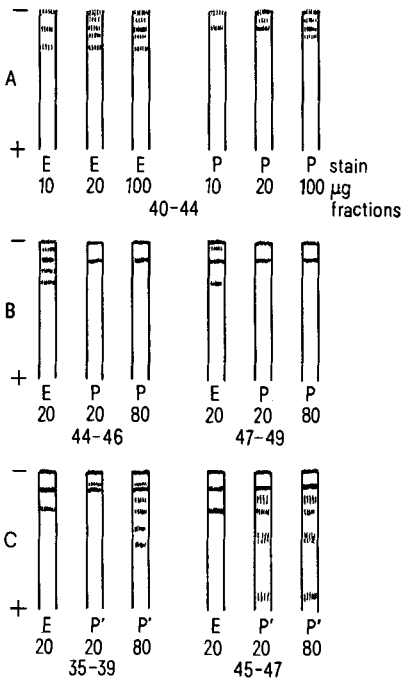

Fig. 2. Polyacrylamide gel electrophoresis patterns of AChE-active fractions from affinity chromatograms

A, **B**, **C** correspond to Fig. 1**A**, **B**, **C**. Start at top of columns. E = enzyme, P = protein stain (P after 1, P′ after 10 days of washing); µg of protein ($E^{1\%}_{280} \equiv 18.0$) applied.

Gel electrophoresis (Fig. 2) provides no indication of striking differences between the active fractions obtained by PTA and Aca-choline chromatography. Two to 3, perhaps 4 active bands are observed. This agrees with the findings of earlier workers

[5], [6], [7]. *Dudai et al.* [6] believe that the multiplicity is due to different states of subunit aggregation. There appears also to be no difference between various 'early' and 'late' active fractions from the Aca-choline columns (Fig. 2, **B**, **C**). We haven't investigated whether the 'early' AChE fractions might indicate a 'leaking' of AChE from the lowaffinity ligand ($K_i \approx 10^{-4}$), or, perhaps, be due to hydrolytic cleavage of ligand plus spacer from the sepharose, as observed for sepharosyl-oxycarbimino-ε-aminocaproylaminoethylthio-3',5'-*cyclic* AMP by *Tesser, Fisch & Schwyzer* [13].

Gel electrophoresis does reveal, however, the presence of other proteins, devoid of AChE activity. Like AChR from *Torpedo* electroplax [17], they are only weakly stained with Coomassie Brillant Blue and are electrophoretically more mobile. A specific affinity for ACh and especially DACh remains to be established.

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