250. ε-Aminocaproylcholine: Chemical Synthesis, Biological Properties, and Interactions with Receptor Molecules[1])

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Summary

ε-Aminocaproylcholine, [$^{14}CH_3$-]ε-aminocaproylcholine, and their Sepharose-2B derivatives (**6**) were synthesized. ε-Aminocaproylcholine is a full cholinergic agonist (nicotinic) with a potency intermediate between that of acetylcholine and of carbachol. ε-Aminocaproylcholine is not hydrolysed by acetylcholine esterase, but is an inhibitor. Its Sepharose-2B derivatives are shown to be effective affinity-chromatographic agents for the isolation of acetylcholine-binding proteins (receptors).

Introduction. – Acetylcholine-sensitive tissues of the nicotinic type are a source of acetylcholine-binding proteins that can be solubilized without immediately losing their specificity. They retain, at least for some time, their specific affinity for acetylcholine, acetylcholine antagonists, and a number of neurotoxins from snake venom, including α-bungarotoxin and α-cobratoxin. Antagonists and toxins have been used as affinity-chromatographic ligands to isolate and purify acetylcholine-binding proteins that are believed to contain or to be identical with the nicotinic acetylcholine receptor [1]. The technique of affinity partitioning [2] appears to be equally well or even better suited for the purpose.

It would be intellectually more pleasing to use a cholinergic agonist as an affinity ligand in both techniques in place of an antagonist or a toxin, or even a partial agonist, because the isolation procedure would then be most specifically directed towards the acetylcholine-binding sites (antagonists and neurotoxins could *a priori* have a different specificity as is shown by their non-competitive displacement with agonists, see below *sub:* 'Association with acetylcholine receptors'). The

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Abbreviations: Acetylcholine: ACh; ε-aminocaproylcholine: AcaCh; acetylcholine esterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7): AChE; acetylcholine-binding protein: AChBP; acetylcholine-binding sites: AChBS; acetylcholine receptor: AChR. BOC-: *t*-butoxycarbonyl.

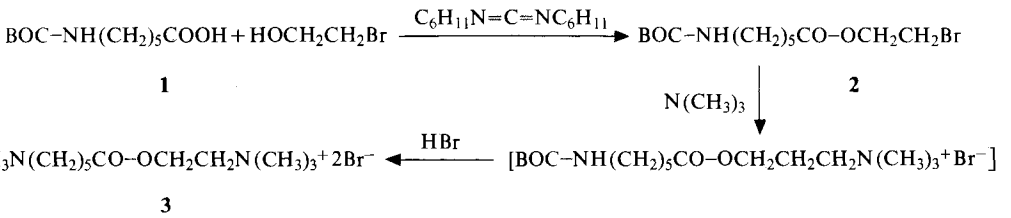
difficulty is, however, that the binding proteins are associated with acetylcholine esterase that will hydrolyse many agonists.

We here describe two syntheses of ε-aminocaproylcholine, including [$^{14}CH_3$-]ε-aminocaproylcholine, and demonstrate that the compound is a full nicotinic agonist with a potency higher than that of carbachol (carbamylcholine) and resistent to the action of acetylcholine esterase. That the compound is promising as an affinity ligand was proved by the isolation of acetylcholine-binding proteins *via* affinity chromatography on the sepharose-linked ε-aminocaproylcholine, **6** (preliminary account of the affinity-chromatographic separation of acetylcholine esterase [3], see also [4]). The isolated binding proteins appear to be similar to the 'acetylcholine receptors'. However, to prove the exact nature will require a great deal more experimentation than has been possible in this work.

Results. – *O*-(6-aminohexanoyl)-choline (ε-aminocaproylcholine) bromide hydrobromide (**3**) was synthesized by two routes. The one depicted in *Scheme 1* was devised analogously to the synthesis of diazoacetylcholine [5]. The yields of the nucleophilic substitution with trimethylamine were invariably poor, practically excluding the introduction of a radioactive label in the CH_3-group by this method.

Scheme 1

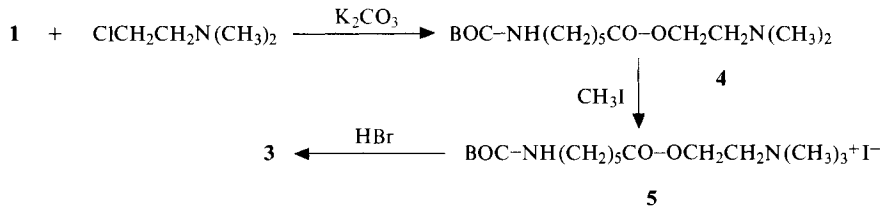
(overall yield of **3** from BOC-6-aminohexanoic acid ≃ 11%)



A considerable improvement was achieved by nucleophilic substitution of 2-chloro-*N*, *N*-dimethyl-ethylamine with *N*-*t*-butoxycarbonyl-6-aminohexanoic acid anion in anhydrous ethyl acetate in the presence of potassium carbonate according to *Scheme 2*. The introduction of a radioactive label (*e.g.* with [^{14}C]CH_3**I** in step **4** → **5**) becomes especially advantageous.

Scheme 2

(overall yield of **3** from BOC-6-aminohexanoic acid ≃ 80%)



As an example for the use of **3** as a ligand for affinity chromatography, the compound was attached to a modified Sepharose 2B containing a '27-atom spacer' according to *Berman & Young* [6]. Radioactive **3** allowed the determination of the number of ligand molecules introduced. Typically, the degree of substitution was voluntarily varied between 1 μmol and 4 mmol of ligand per ml of substituted Sepharose 2B. The latter was tentatively assigned the structure of *O*-[32-(*O*-sepharosyl-isoureido)-8,11,21,24-tetraoxo-7,12,16,20,25,29-hexaazadotriacontanoyl]-choline:

6 Seph-OC(=NH)-[$NH(CH_2)_3NH(CH_2)_3NH$-$COCH_2CH_2CO]_2$-$NH(CH_2)_5CO$-$OCH_2CH_2N(CH_3)_3^+X^-$

Of course, substitutions of other macromolecular solids [7] by **3**, including those used in the technique of affinity partitioning [2] can be envisaged.

Acetylcholinesterase (AChE) does not hydrolyse AcaCh (**3**). The enzyme used in these studies[1]) had been purified from electric organs of *Torpedo marmorata* [4]. Its K_m for ACh iodide was about 0.11 mM. AcaCh is a non-competitive inhibitor with $K(\text{inh}) \simeq 0.1$ mM [8].

Biological activity [8]. In the frog *rectus abdominis* muscle preparation (nicotinic receptors), AcaCh behaves as a full agonist: maximal effects are the same as those of ACh and of carbachol (carbamoylcholine). Its potency is intermediate; the concentrations required for half-maximal stimulation were determined from logarithmic dose/response curves and were found to be for ACh about 0.3 μM, for AcaCh about 5 μM, and for carbachol about 14 μM. *Hill* plots suggest 'positive cooperativity' ($n > 1$) in the same order of magnitude for both AcaCh and carbachol.

Association with acetylcholine receptors [8]. The purified receptor protein (AChR)[2]) had been isolated from the electric organ of *Torpedo marmorata* by affinity chromatography on α-cobratoxin [9]. [^{125}I]α-Cobratoxin displacement experiments were performed both according to *Schmidt & Raftery* [10] (10 min incubation with [^{125}I]α-cobratoxin) and *Fulpius* [11] (15 h incubation). Especially with prolonged incubation, carbachol and AcaCh give non-linear *Lineweaver-Burk* and *Scatchard* plots; only *d*-tubocurarine shows a behaviour corresponding to a homogeneous or non-cooperative set of 'binding sites'. With the *Schmidt-Raftery* method and the *Michaelis-Menten* approximation an apparent inhibition constant of 3 μM ± 0.5 μM was found for AcaCh. This value is virtually identical with the concentration required for half-maximal stimulation in the frog muscle test (5 μM).

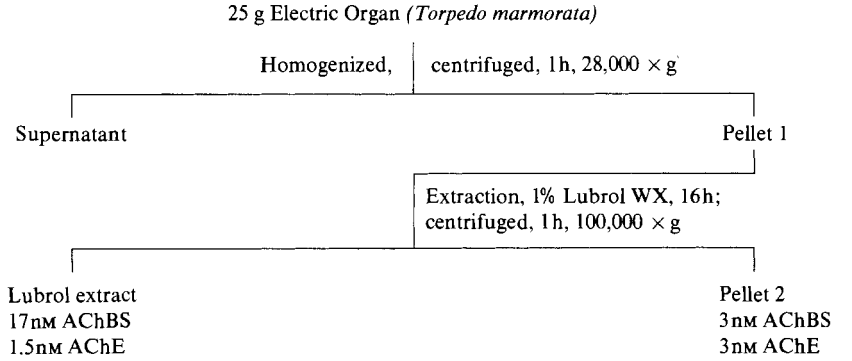
In the experiments according to *Fulpius*, very different values were found, as anticipated [11]. The *Scatchard* plots for AcaCh and carbachol can be interpreted on the basis of two receptor site populations. The following apparent inhibition constants were thus found [8]: $K(\text{inh}) \simeq 0.7$ nM and 7 nM (AcaCh); $K(\text{inh}) \simeq 0.6$ nM and 4 nM (carbachol); $K(\text{inh}) \simeq 1.7$ nM (*d*-tubocurarine; only one value derived from the strictly linear *Lineweaver-Burk* and *Scatchard* plots).

[1]) Gift of PD Dr. *W. Hopff*, Pharmakologisches Institut der Universität Zürich.

[2]) Gift of Prof. Dr. *B. Fulpius*, Dépt. de Biochimie, Sciences II, Université de Genève.

Affinity chromatography of acetylcholine binding proteins [12]. The starting material, containing solubilized AChBP, was prepared according to *Scheme 3* [13–15].

Scheme 3



The Lubrol extract was found to contain per g of tissue 0.68 nM or $4.2 \cdot 10^{14}$ AChBS (by equilibrium dialysis) and 3.4 mg of protein (estimated with the methods of *Lowry et al.* [16] and *Rosenberry et al.* [17]). These figures compare favorably with those reported by *O'Brien & Eldefrawi* [13] (0.65 nM/g and 3.1 mg/g). On the basis of neurotoxin binding studies, $6.6 \cdot 10^{14}$ and $2.4 \cdot 10^{14}$ binding sites per g of electric organ have been found for *Torpedo* [18] and for *Electrophorus* [19], respectively. The ratio of AChE/AChBS is 0.24; *O'Brien & Eldefrawi* [13] estimated 0.3.

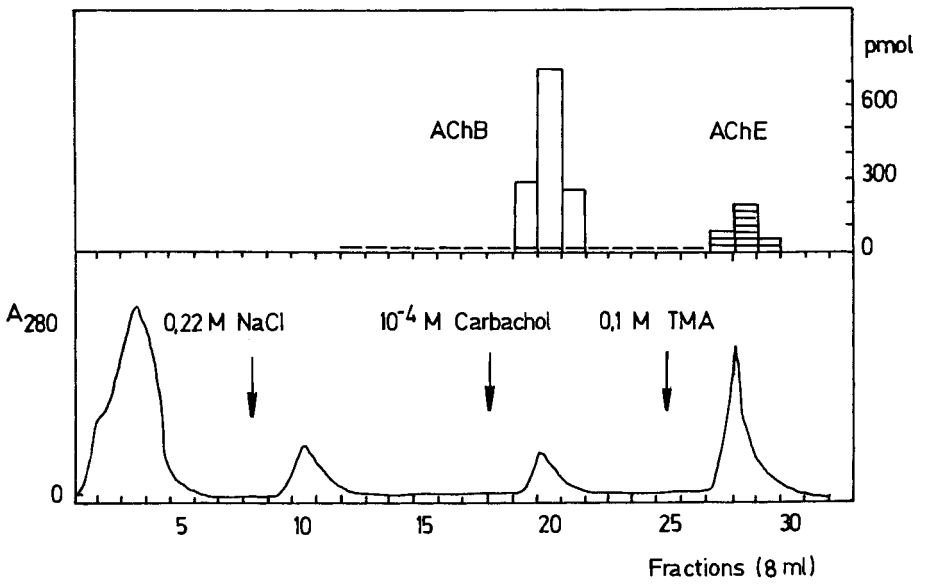


Figure. *Affinity chromatographic separation of solubilized proteins from* Torpedo marmorata *electric tissue* (details see experimental part). TMA = tetramethylammonium bromide.

The chromatographic profile of a typical separation with **6** is shown in the *Figure*. Inactive components were eluted with the buffer alone (fractions 1–7) and after adding NaCl to 0.22 N (fractions 8–17). Carbachol (10^{-4} M) elutes AChBP (fractions 19–21), and 0.1 M tetramethyl-ammonium bromide, finally, the main quantity of AChE (47%) in fractions 27–29. However AChE leakage is observed already after enhancing the ionic strength (fractions 12–26, at a rate of about 20–25 pmol per fraction).

The crude and chromatographically purified proteins lose their binding capacity with a half-life of about 2 days. Similar observations have already been made with respect to neurotoxin binding [15] [20]. Taking such losses into account, logarithmic dose/association curves suggest apparent association constants between ACh and crude as well as chromatographically purified AChBP of $K(\text{ass}) \simeq 10^7$–$10^8$ [12].

The results of the affinity-chromatographic purification according to the *Figure* are summarized in *Table 1*. The binding proteins appear to be purified ten-fold. The ratio of AChBS/AChE increases about seven-fold. The yields (about 15–20%) are smaller than those reported using α-bungarotoxin, *e.g.* from *Electrophorus* (30–50%) [21] and *Torpedo californica* (32%) [18], but similar to the yields obtained by electrofocussing techniques [15]. The number of AChBS found in this preparation is on the low side, but comparable to those reported *(Table 2)*.

Table 1. *Data of the affinity-chromatographic separation shown in the Figure*

	Proteins (total, mg)	AChBS[a]) pmol ACh bound		AChE		Ratio AChB/AChE
		total	per mg	pmol[b])	U/mg	
Lubrol extract, 10 ml	40 ± 4	8000 ± 400	200 ± 30	680	42	~12
Fractions 19–21	0.8 ± 0.1	1280–1600	1400–2400	16	50	~80
Fractions 27–29	2.6 ± 0.3			320	310	

[a]) Apparent association constants about 10^3–10^4 times greater than those of AChE.
[b]) Assumed mol-wt. 260,000 [25].

Table 2. *Comparison of some AChBS and neurotoxin-binding site yields after purification with affinity chromatography*

Electric tissue	Binding assay[a])	Homogenate (pmol/mg)	Extract (pmol/mg)	Purified (pmol/mg)	Reference
Electrophorus el.	[^{3}H]α-Bgt	1.5	15	2000	[21]
Torpedo californ.	[^{125}I]α-Bgt	170	320	6600	[18]
Torpedo marmor.	[^{125}I]α-Ctx			3300/7800	[22]
Torpedo marmor.	[^{3}H]ACh	100	200	-	[15]
Torpedo marmor.	[^{3}H]ACh	-	200	1400–2400	this work

[a]) Bgt = bungarotoxin; Ctx = cobratoxin *(Naja naja siamensis)*.

Experimental Part

General remarks. – The 'usual isolation procedure' referred to in the examples comprises: partition between a specified organic and various aqueous phases (acidic, basic, neutral, depending on the nature of the desired product and the by-products to be removed), drying with conc. NaCl-solution, Na_2SO_4, and evaporation of the organic solvent. Evaporation was always performed in a rotary evaporator at low temperature and *in vacuo*. Ratios of solvents, especially for TLC. on silica gel are in volume parts (*v/v*); m.p. were determined in open capillaries and are uncorrected.

N-t-*Butoxycarbonyl-6-aminohexanoic acid* (**1**). A solution of 13.1 g (0.1 mol) ε-aminocapronic acid in 200 ml dioxane/water 1:1 was treated with 15.5 g (0.108 mol) *t*-butoxycarbonyl azide. The pH was raised to 10.5 and held constant by addition of 2N NaOH. The reaction was complete after 5 h at 20°. The dioxane was evaporated and the aqueous residue subjected to the usual isolation procedure with ethyl acetate. Recrystallization from hexan/diisopropylether yielded 20 g (86%) **1**, m.p. 38–39°.

$C_{11}H_{21}NO_4$ (231)	Calc.	C 57.12	H 9.15	N 6.06	O 27.67%
	Found	„ 57.06	„ 9.07	„ 5.99	„ 27.75%

*β-Bromoethyl (*N-t-*butoxycarbonyl)-6-aminohexanoate* (**2**). A solution of 11.2 g (48.5 mmol) **1** and 5 g (24.3 mmol) dicyclohexylcarbodiimide in 200 ml of toluene containing 5% pyridine was stirred at 0° for 30 min. The precipitate of dicyclohexylurea was filtered off and the filtrate treated with 4 g (32 mmol) of 2-bromoethanol. After 15 h at 20° the crude product was isolated by the usual procedure (ethyl acetate). It was chromatographed over silica gel (3×26 cm) with $CHCl_3$: 6.8 g (83%) **2**, colourless oil. – 60-MHz-NMR. (DMSO): 4.4 (*t*, 2 H); 3.5 (*t*, 2 H); 3.3–2.9 (*m*, 2 H); 2.5–2.1 (*m*, 2 H); 1.9–1.1 (*m*, with the $-CH_3$ signal of BOC at 1.45, 15 H).

$C_{13}H_{24}BrNO_4$ (338)	Calc.	C 46.16	H 7.15	Br 23.63	N 4.14	O 18.92%
	Found	„ 46.24	„ 7.23	„ 23.39	„ 4.12	„ 18.90%

O-*(6-aminohexanoyl)-choline bromide hydrobromide* (**3**). Trimethylamine in the gas phase (dried over NaOH) was passed into a solution of 2 g (5.9 mmol) **2** in 20 ml dry nitrobenzene at 0° until a volume of 28 ml was attained. The solution was kept at 20° under pressure for 10 days. The somewhat milky mixture was filtered and the trimethylamine removed from the filtrate by evaporation. The residue was treated with 40 ml of dry ether and cooled to −25°. The crude, crystalline *O*-(*N*-*t*-butoxycarbonyl-6-aminohexanoyl)-choline bromide (690 mg) was used without further purification: 60 mg were treated at 0° with 0.1 ml hydrobromic acid (48%). After 2 min 20 ml of water/*t*-butyl alcohol 1:1 were added and the solution lyophilized. The viscous residue was crystallized from 2-propanol and dried over P_2O_5: 25 mg (13%), m.p. 127–129°. – 60-MHz-NMR. (DMSO): 8.2–7.7 (*m*, 3 H); 4.7–4.2 (*m*, 2 H); 3.9–3.5 (*m*, 2 H); 3.15 (*s*, 9 H); 2.9–2.2 (*m*, with signals from DMSO); 1.8–1.3 (6 H).

$C_{11}H_{26}Br_2N_2O_2$ (378)	Calc.	C 34.93	H 6.93	Br 42.27	O 8.46	N 7.41%
	Found	„ 34.85	„ 6.93	„ 42.31	„ 8.55	„ 7.50%

N-*Dimethyl*-O-*(*N-t-*butoxycarbonyl-6-aminohexanoyl)-colamine* (**4**). To a solution of 2.31 g (10 mmol) **1** and 5.76 g (40 mmol) 1-dimethylamino-2-chloroethane (recrystallized from ethanol) in 100 ml ethyl acetate were added 2.76 g (20 mmol) of finely ground, anhydrous K_2CO_3 and the mixture was boiled under reflux for 5 h. Insoluble material was removed by filtration and the filtrate subjected to the usual isolation procedure (ethyl acetate, $NaHCO_3$ and saturated aq. NaCl-solution). The yield of crude product was 3 g (about 100% with respect to **1**). It appeared to be pure (TLC.), but the ester linkage was quite labile upon storage. For this reason, the product was not further identified, but used directly in the next step. TLC.: 0.45 ($CHCl_3$/MeOH 4:1); 0.3 (BuOH/AcOH/H_2O 10:1:3).

O-*(*N-t-*Butoxycarbonyl-6-aminohexanoyl)-choline iodide* (**5**). A solution of 3.02 g (10 mmol) of crude **4** and 1.3 g (11 mmol) CH_3I in 50 ml acetone was refluxed for 15 h. A slight amount of insoluble material was removed by filtration and the clear filtrate evaporated. The yellow, oily residue was dissolved in water and extracted with ethyl acetate to remove impurities. The now colourless aqueous phase was lyophilized and the residue recrystallized from 2-propanol/diisopropyl ether. Yield 3.8 g (86%) colourless crystals, m.p. 94.5°. TLC.: 0.19 ($CHCl_3$/MeOH 4:1); 0.215 (BuOH/AcOH/H_2O 10:1:3); 0.42 (BuOH/AcOH/H_2O 5:2:3). – IR. (Nujol): 3300, 1735, 1680, 1150 cm^{-1}. – 360-MHz-NMR.

(DMSO): a) 1.37 (15 H); b) 2.33 (2 H); c) 2.92 (2 H); d) 3.17 (9 H); e) 3.73 (2 H); f) 4.47 (2 H); g) 6.7 (1 H). –

$$(CH_3)_3COCONHCH_2(CH_2)_3CH_2COOCH_2CH_2N(CH_3)_3^+ I^-$$
a g e a b f c d

$C_{16}H_{33}IN_2O_4$ (444)	Calc.	C 43.24	H 7.49	I 28.56	N 6.30%
	Found	„ 43.18	„ 7.44	„ 28.58	„ 6.15%

O-*(6-aminohexanoyl)-choline bromide hydrobromide* (**3**). A solution was made with 2.2 g (5 mmol) **5** and dilute acetic HBr-solution. After 10 min at 20° ether was added to precipitate the crude **5** as an oil. This was dissolved in water, extracted with $CHCl_3$ and ethyl acetate to remove impurities, and the aqueous phase lyophilized. The residue was dissolved in 2-propanol/MeOH/ 5:1, and treated with a few drops of diisopropyl ether. After 20 h the colourless crystals were isolated: 1.76 g (93%), m.p. 128°. TLC.: 0.17 (BuOH/AcOH/H_2O 5:2:3). – IR. (Nujol): 3350, 1715, 1600, 1180 cm^{-1}. – 360-MHz-NMR. (DMSO): a) 1.47 (6 H); b) 2.32 (2 H); c) 2.73 (2 H); d) 3.20 (9 H); e) 3.77 (2 H); f) 4.47 (2 H); g) 7.03 (3 H). –

$$^+H_3NCH_2(CH_2)_3CH_2COOCH_2CH_2N(CH_3)_3^+\ 2\ Br^-$$
g c a b f e d

$C_{11}H_{26}Br_2N_2O_2$ (378)	Calc.	C 34.93	H 6.93	Br 42.27	N 7.41%
	Found	„ 34.78	„ 6.90	„ 42.04	„ 7.22%

[^{14}C-Me] O-*(6-aminohexanoyl)-choline chloride hydrochloride.* A solution of 1 g (3.31 mmol) **4** and 1.71 g (12.09 mmol) [^{14}C] CH_3I with a specific activity of 55 mCi/mmol) in 30 ml CH_2Cl_2 was refluxed at 50–60° for 20 h. The solvent was evaporated and the identity of the residual oil as [^{14}C] **5** was ascertained by TLC. and NMR. This product was dissolved in 1.2 eq. of 0.1N HCl in formic acid and kept for 10 min at 20°. The solvent was evaporated and the residual oil dried in a desiccator over solid NaOH. The product was repeatedly recrystallized from 2-propanol/diisopropyl ether and ethanol/ diisopropyl ether/diethyl ether. The product was characterized by TLC. and NMR. as **3** (without specification of the anions; these were shown to be Cl^- in the usual manner with $AgNO_3$). Yield overall 75%, specific activity 2.644 mCi/mmol.

O-*[32-(O-Sepharosyl-isoureido)-8,11,21,24-tetraoxo-7,12,16,20,25,29-hexaaza-dotriacontanoyl]-choline* (**6**). The pH of a suspension of 80 ml Sepharose 2B *(Pharmacia)* at 20° was held constant at 11 by addition of 5N NaOH while 15 g of solid bromocyanogen *(Pierce Chemical)* were slowly added. After 30 min the gel was quickly washed at 0° with 2 l of 0.1M $NaHCO_3$-buffer (0.1M, pH 10) and then treated with a cold (4°) solution of bis (3-aminopropyl)amine (diaminopropyl-amine, *Aldrich*) in 3000 ml water (pH 10). The suspension was stirred overnight. The gel was then washed with water, suspended in 300 ml water (4°) and treated with small portions of a total of 18 g of succinic anhydride *(Fluka)*, the pH being held at 6 with 5N NaOH. The suspension was stirred for at least 6 h at 4° and then washed and suspended in 150 ml of water. Bis(3-aminopropyl)amine (20 g) dissolved in 100 ml of water and adjusted to pH 5, and 25 g of 1-cyclohexyl-3-[2-(*N*-methylmorpholino)-ethyl]-carbodiimide *p*-toluenesulfonate *(Aldrich)* were added. The suspension was stirred overnight at RT. (~20°). The resulting gel was then again washed and treated with 18 g of succinic anhydride as above, and finally washed with water.

A suspension of 20 ml of this gel in 40 ml of water was treated with 100 mg **3** and 2.5 g of 1-cyclohexyl-3-[2-(*N*-methylmorpholino)-ethyl]-carbodiimide *p*-toluenesulfonate at about pH 5 and 20° overnight. The gel was thoroughly washed with 0.1M sodium acetate buffer (pH 5), 0.1M $NaHCO_3$-buffer (pH 10) and water.

The compounds were stored in 0.1M sodium acetate (pH 5) with 0.1% sodium azide at 4°.

Preparation of solubilized binding proteins. All operations were performed at 4°. The fresh electrical organ of *Torpedo marmorata* (Arcachon, France) (25 g) was cut into small pieces and homogenized in 50 ml water for 1 min with a household blender. The suspension was centrifuged at 28,000 × g for 1 h. The supernatant contained about 0.25 nmol of AChE. The pellet (~11 g) was lyophilized and again homogenized for 30 sec. in 20 ml of a 17mM sodium phosphate buffer, pH 7.4, containing 120mM NaCl, 5mM KCl, 1mM $CaCl_2$, and 1.2mM $MgCl_2$. The homogenate was treated with 2.5 ml of a 10% Lubrol

WX solution *(ICI)* to a final concentration of 1% and stirred for 16 h. Centrifugation at 100,000×*g* yielded the supernatant crude extract of solubilized binding proteins.

Equilibrium dialysis. These experiments were performed at 4° in an apparatus according to *Weder et al.* [23] with 1.3 ml cells and cellulose membranes (alternately, 200 µl cells and *Spectrapor 2* cellulose membranes – from *Spectrum Medical Ind. Ltd.* – with an exclusion of 8,000–12,000 molecular weight, were used). The protein solutions of 0.7–1 ml containing 0.01–4 mg of protein per ml were incubated for 15 min previous to the experiment with 0.1 mM *O,O*-diethyl-*S*-(2-diethylaminoethyl)-thiophosphate (Tetram, *ICI*) in order to inhibit AChE activity without imparing binding to AChBP. The dialysis was carried out against a 17mM sodium phosphate buffer, pH 7.4, containing 120mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1.2mM $MgCl_2$, and 0.1mM Tetram, as well as the investigated concentration of [^{3}H]ACh (specific activity 290 mCi per mmol; *The Radiochemical Centre*, Amersham). After dialysis for 10 to 16 h with 12 revolutions per min, samples of 0.1–0.8 ml were withdrawn and processed in a liquid scintillation counter at an efficiency of 25–30%.

Determination of acetylcholinesterase activity. AChE was assayed with the pH-Stat *(Radiometer)* method according to *Kremzner & Wilson* [24]. The assays were performed under nitrogen in solutions of 0.15M NaCl, 0.04M $MgCl_2$, 0.1% bovine serum albumin *(Fluka)* at 25° and pH 7. The titrations were carried out with 0.05N NaOH. The initial concentration of ACh iodide was 1.7mM. 1 unit of enzyme activity represents 1 µmol of ACh iodide hydrolysed per min. Specific activities were calculated from absorbance at 280 nm, using $E^{1\%}_{280} = 18.0$ [17]. Crystalline AChE, mol-wt. 260,000, has 12,500 U/mg [25].

Determination of nicotinic cholinergic activity. Rana esculenta rectus abdominis muscles were used as the test objects and their contraction measured in the usual pharmacological assay system [26]. The bath consisted of 4 ml *Krebs* solution (136mM NaCl, 5.6mM KCl, 16.2mM $NaHCO_3$, 1.2mM NaH_2PO_4, 1.2mM $MgCl_2$, 2.2mM $CaCl_2$, 5.5mM glucose) that was equilibrated before use with a mixture of 95% O_2 and 5% CO_2. The maximal effect was produced by 50µM carbachol (*O*-carbamoylcholine) and was taken as E_{100}. Logarithmic dose/activity curves were established in the usual manner with 5 to 7 different agonist concentrations. Because neither carbachol nor **3** are hydrolysed by AChE, the usual addition of 3 mg/ml of eserin in order to inhibit AChE was omitted except in the experiments involving ACh.

Affinity chromatography of acetylcholine-binding proteins. All operations were performed at 4°. The crude electric organ extract (10 ml) described above, containing 680 pmol AChE and 8000 pmol of AChBS was chromatographed on a 2×7 cm column of 20 ml **6**. Fractionation was effected by the following solvents (a) 48 ml sodium phosphate "buffer III" (17mM, pH 7.4, containing 120mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1.2mM $MgCl_2$, and 1% Lubrol WX); (b) 80 ml buffer III, containing 0.22 instead of 0.12M NaCl; (c) 64 ml buffer III, as in b, but with 0.1mM carbachol *(Sigma)*; (d) 80 ml buffer III, as in b, but with 0.1M tetramethylammonium bromide. The volume of the individual fractions was 8 ml, gathered at a rate of 8 ml per h. Fractions containing AChBP were sometimes concentrated to a smaller volume by ultrafiltration in a "Diaflo" apparatus (*Amicon;* membrane UM-20E). Carbamoylcholine and tetramethylammonium bromide were removed by dialysis against buffer III, 30 ml/h, during 10–11 h in a "Mini-Beaker" of *Bio-Rad.*

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