Properties of the Soluble and Membrane-Bound Forms of Acetylcholinesterase Present in Pig Brain

D. T. Plummer, C. A. Reavill, and C. H. S. McIntosh*

Department of Biochemistry, Chelsea College (University of London), Manresa Road, London, SW3 6LX, England

Approximately 15% of the total acetylcholinesterase (AChE) activity of pig brain cortex can be extracted in dilute buffer solution and the properties of this »soluble« form of the enzyme have been compared with the membrane bound enzyme which was brought into solution by extraction with 1% Triton X-100 or 1 mM EDTA. The activity of the »soluble« enzyme against a range of substrates is identical to the membrane enzyme. The variation of activity with pH and substrate concentration are similar for the two physical forms of the AChE. Gradient polyacrylamide gel electrophoresis demonstrated the similarities of the »soluble« and detergent solubilized enzyme preparations. Three molecular weight species were common to both preparations: 353 000, 262 000, and 68 000 and in addition the »soluble« enzyme had a band of mol. wt. 135 000 while the Triton X-100 extract contained species of mol. wt. 181 000 and 83 000.

The membrane AChE showed a break in the Arrhenius plot with a transition temperature of 27 °C and this was abolished with detergent. In contrast the »soluble« enzyme showed no break in the Arrhenius plot suggesting the absence of associated membrane material. There are however more similarities than differences between the two physical forms of the enzyme which appear to be closely related.

INTRODUCTION

In all species so far studied, acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7.) has been shown to be a membrane-bound enzyme[1,2] although its exact relationship with the membrane has not been fully clarified[3,4]. Most information on the pure enzyme has been obtained from *Electrophorus electricus* which is a very rich source of acetylcholinesterase (AChE). The enzyme from electric eel is relatively easy to solubilize and subsequently purify especially since the application of affinity chromatography[5]. The enzyme from mammalian brain however is tightly bound to the membrane and a number of different methods have been applied to bring the enzyme into solution. In particular, the use of the non-ionic detergent Triton X-100 has proved to be most useful[6,7] and more recently repeated extraction of brain homogenate with low ionic strength media containing EDTA has also been used[8,9]. McIntosh and Plummer[10] have compared several methods for bringing the enzyme into solution and have reported on the effect of the solubilization method on the

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* Present address: 34 Göttingen, Kliniken der Universität Göttingen, Hormon Labor, Humboldtaller, W. Germany.

number and distribution of the multiple molecular forms of acetylcholinesterase from pig brain. These workers showed that about 14% of the AChE was soluble in aqueous solution without further treatment and this present paper is concerned with comparing the properties of this »naturally soluble« part of the brain acetylcholinesterase with the 86% which is membrane bound. The effect of solubilization by detergent and also by EDTA extraction on the properties of the membrane enzyme has also been investigated.

A knowledge of the molecular forms of the soluble and membrane-bound acetylcholinesterase of brain and their relationship to each other should give a clearer insight into some of the molecular mechanisms of synaptic transmission.

MATERIALS AND METHODS

Chemicals

Reagents were obtained as follows: 5,5'-dithiobis-(2-nitrobenzoate) (DTNB), bovine serum albumin, acetylthiocholine iodide, (ATChI) acetylcholine iodide (AChI) and catalase from Sigma (London): EDTA and sucrose from Fisons (Loughborough, Leicestershire). Triton X-100 was obtained from BDH Chemicals Ltd (Poole, Dorset) and Carbowax 20 M purchased from G. T. Gurr and Co Ltd (London). The acetylcholinesterase inhibitor BW 284 C51 was obtained from Wellcome Reagents Ltd (Beckenham, Kent).

All other reagents were analytical grade and all solutions were prepared in glass distilled water redistilled from an all glass still.

Solubilization of acetylcholinesterase

All solubilization procedures were performed on fresh porcine brains. The brain cortex was excised then homogenized in a Waring blendor for 5 min at 4 °C followed by centrifugation in the cold in an M. S. E. ultracentrifuge S.S.65 or S.S.50.
Preparation of »soluble« enzyme. A 20% w/v homogenate of the cortex was prepared in 0.03 M sodium phosphate buffer, pH=7.0. The homogenate was then centrifuged at 100 000 g for 1 hour and the acetylcholinesterase in the supernatant taken to be the 'soluble' enzyme.

Solubilization with Triton X-100. Acetylcholinesterase was essentially solubilized by the method of Ho and Ellman[7]. The cortex was blended as above at 20% w/v, centrifuged at 100 000 g for 1 hour and the supernatant removed. The pellet was resuspended in the same volume of buffer containing 1% Triton X-100 w/v. The mixture was stirred for 10 minutes at room temperature and the suspension clarified by centrifuging at 100 000 g for 1 hour. The activity in the supernatant was taken as the Triton solubilized AChE.

Solubilization with EDTA. Extraction with EDTA was accomplished by modifying the method of Chan *et al.*[8] A 20% w/v homogenate was prepared in 0.03 M sodium phosphate buffer pH=7.0 and centrifuged at 100 000 g for 1 hour. The pellet was resuspended to the original volume in a solution containing 0.03 M phosphate buffer and 10^{-3} M EDTA. This suspension was stirred for 2 hours at 4 °C, recentrifuged at 100 000 g for 1 hour and the supernatant removed. Two more extractions of the homogenate were carried out and the 3 supernatants combined and concentrated with Carbowax.

Other methods of solubilization. Other methods used including extraction with organic solvent, enzyme treatment and ultrasonication were caried out as previously described.[10]

Assay of acetylcholinesterase

pH-stat. Most of the assays of AChE were carried out at 30 °C using a pH-stat (Radiometer, Copenhagen, Denmark). The reaction mixture consisted of NaCl (0.15 M), $MgCl_2$ (1.3 mM), acetylcholine iodide (1 mM) and enzyme preparation (0.3—1.0 ml) made up to a final volume of 8.0 ml. The pH was maintained at 7.9 by the automatic

titration of 20 mM NaOH to neutralize the H^+ released from the hydrolysis of the substrate. The spontaneous H^+ release was measured for 5 min before the addition of the substrate and any appropriate correction made. Enzyme activities were expressed as μmoles/min/mg protein.

Colorimetric method. For the measurement of a large number of samples, the colorimetric method of Ellman *et al.*[11] was used to assay AChE. All spectrophotometric measurements were made at 30 °C in a Perkin-Elmer 124 spectrophotometer or Pye Unicam S.P. 800 spectrophotometer. The reaction mixture was composed of 2.83 ml of 0.1 M sodium phosphate buffer, pH=8.0, 0.05 ml of enzyme preparation, 0.1 ml of 5,5'-dithiobis-(2-nitrobenzoate) and 0.02 ml of acetylthiocholine iodide added in that order. The hydrolysis of acetylthiocholine produces thiocholine which reacts with DTNB to give 5-thio-2-nitrobenzoate which has an extinction maximum at 412 nm.

Protein

The method of Lowry *et al.*[12] was used with crystalline bovine serum albumin as standard.

Gradient polyacrylamide gel electrophoresis

Electrophoresis was carried out on a concave gradient of polyacrylamide (4—24%) and the gels stained for AChE activity as previously described[10].

Sucrose-gradient centrifugation

Linear sucrose gradients were prepared and centrifuged according to the method of Martin and Ames[13]. The centrifuge tubes contained 5—20% w/v linear sucrose gradients (19 ml) on a cushion of 60% sucrose (2 ml). The gradients were stored at 4 °C for 5 hours then 0.5 ml of enzyme preparation together with a catalase marker was carefully overlaid onto the sucrose. The tubes were then centrifuged at 100 000 *g* for 17 hours at 4 °C in a 3×25 ml swing out rotor. Fractions (0.5 ml) were then taken from the centrifuge tubes using an M. S. E. tube piercer and assayed for AChE activity using the Ellman method.

Arrhenius plots

The activity of the enzyme preparation (*v*) was determined over a range of substrate concentration (*s*) up to 1 mM acetylcholine iodide. The maximum enzyme activity (V_{max}) was then determined from these results by plotting s/v against *s* or 1/v against 1/s. The temperature used ranged from 5 °C to 50 °C over which the enzyme was found to be quite stable during the time of assay. Log V_{max} was then plotted against the reciprocal of the absolute temperature.

<div align="center">RESULTS</div>

Solubilization of acetylcholinesterase

Pig brain cerebral cortex contains a »naturally soluble« form of AChE and this was clearly shown when a 20% brain homogenate in dilute buffer was centrifuged for 1 hour at 100 000 *g*. Under these conditions, 15% of the AChE activity of the homogenate was recovered in the supernatant. This is referred to as the »soluble« or »naturally soluble« form of the enzyme.

Treatment with detergents was found to be the most efficient way of bringing the rest of the AChE into solution and Triton X-100 at a concentration of 1% w/v solubilized about 65% of the activity and an increase in the detergent concentration to 2% made only a marginal improvement in the amount of enzyme brought into solution. Previous reports had shown that

Triton X-100 with high salt concentration solubilized almost all of the AChE present in human erythrocytes[14] and this was shown to be the case for the brain enzyme (Fig. 1). However, low concentrations of KCl suppressed the solubilization by detergent and concentrations above 0.6 M caused the separation of lipid material which was difficult to remove. A standard procedure was therefore adopted of using only 1% Triton X-100 with no KCl present. Treatment with lysolecithin was also very efficient giving 85% of the enzyme in the soluble form but the method could not be adopted on a large scale because of the expense involved.
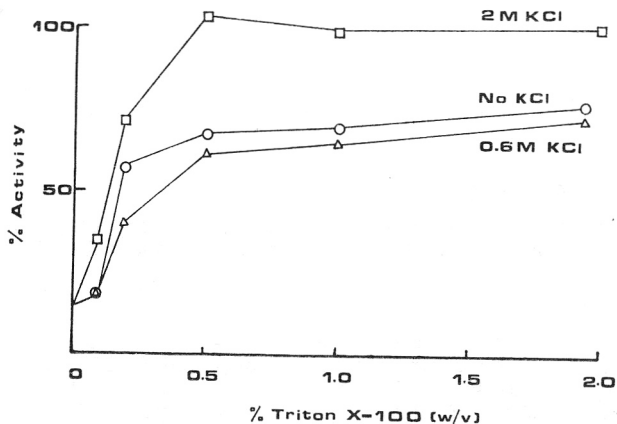

Fig. 1. Solubilization of brain acetylcholinesterase with Triton X-100 and KCl. The activity in the supernatant is plotted on the ordinate.

The other methods examined briefly were relatively ineffective at bringing the enzyme into solution and with some treatments considerable loss of activity was observed (Fig. 2).

Kinetic studies on soluble and membrane acetylcholinesterase

The standard conditions for assay were predetermined on samples of brain homogenate and enzyme solubilized with Triton X-100. Maximum activity was observed over the pH range 7.9 to 8.5 for both the membrane and solubilized preparations. Non-enzymic hydrolysis of AChI was zero up to pH = 8 but started to become significiant above this point, so all assays were carried out at pH = 7.9.

Optimum activity was obtained at 1 mM substrate concentration for the homogenate and 2 mM for the Triton X-100 extract (Fig. 3). This led to the adoption of 1 mM for all standard assays. Both preparations showed inhibition by high substrate concentrations but the solubilized enzyme was more sensitive to inhibition giving only 65% of the maximum activity at 10 mM substrate concentration compared with 85% for the homogenate. The other difference observed was in the Michaelis constants but this was only small. The solubilized enzyme had a K_m of 69 µM and the membrane enzyme gave a slightly higher value of 80 µM. The 'naturally soluble' form however had a higher K_m of 220 µM.
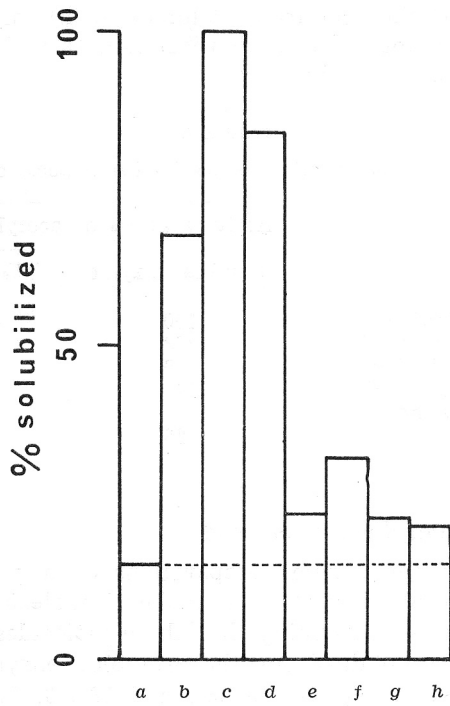
Fig. 2. Solubilization of the acetylcholinesterase of pig brain cortex.
a) Dilute buffer extract; b) 1% Triton X-100; c) 1% Triton X-100/1 M KCl; d) Lysolecithin, 12 mm;
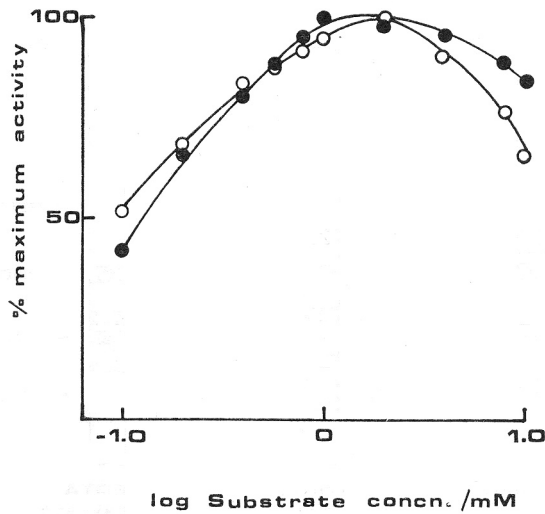e) Butanol extract; f) EDTA, 1 mM; g) Nagarse; h) Ultrasonication.

Fig. 3. The substrate concentration dependence of (●) membrane bound and (○) Triton X-100
solubilized acetylcholinesterase of pig brain.

The specificity of the »naturally soluble« AChE and the Triton X-100 extracted enzyme were found to be identical using a wide range of substrates at optimum concentration (Table I).

TABLE I.

Hydrolysis of various choline esters by brain acetylcholinesterase

Substrate	Activity as % of acetylcholine iodide	
	Soluble enzyme	1% Triton extract
Acetylcholine iodide	100	100
Propionylcholine iodide	83	84
Butyrylcholine iodide	0	0
Acetylthiocholine iodide	149	146
Acetyl-β-methylcholine bromide	20	22
Tributyrin	4	5

Multiple forms of acetylcholinesterase

Electrophoresis of the enzyme preparations on a gradient of polyacrylamide showed that the AChE exists in a number of molecular forms of differing molecular weight (Fig. 4). Assuming that these molecules are spherical, then the molecular weights obtained for the »soluble« enzyme after calibration of the gels were 340 000, 260 000, 135 000 and 68 000. The Triton-solubilized enzyme had three bands in common with the 'soluble' enzyme with mol. wt. values of 365 000 and 264 000 and 68 000. In addition to these species, a band of mol. wt. 181 000 and a faint band of 83 000 were observed. Extraction with EDTA again gave one band whose mol. wt. (250 000) was similar to those found in the other preparations. However in this case, lower mol. wt. zones
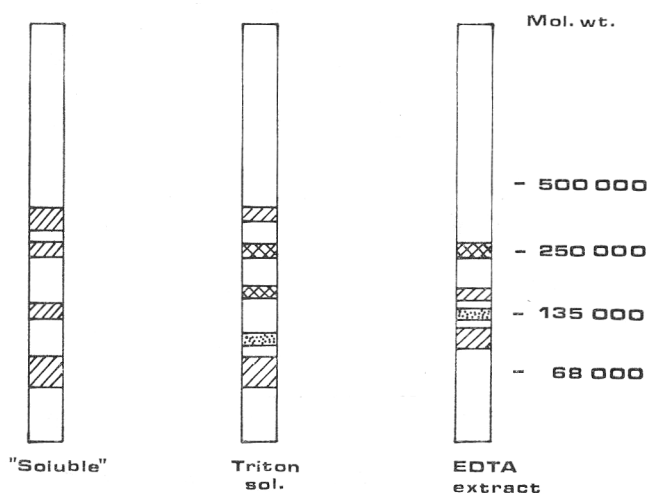

Fig. 4. Electrophoresis of soluble and solubilized acetylcholinesterase on a gradient of polyacrylamide.

were observed corresponding to mol. wt. of 160 000 and 84 000 together with a faint zone of mol. wt. 124 000.

The one feature in common is the presence of a species whose mol. wt. is in the region of 250 000 and this was the major species found after sedimentation on a sucrose gradient. The sedimentation constant lay in the region 11 to 12 S and in the case of the detergent solubilized enzyme this was the only species detected and was unaffected by high ionic strength (Fig. 5).
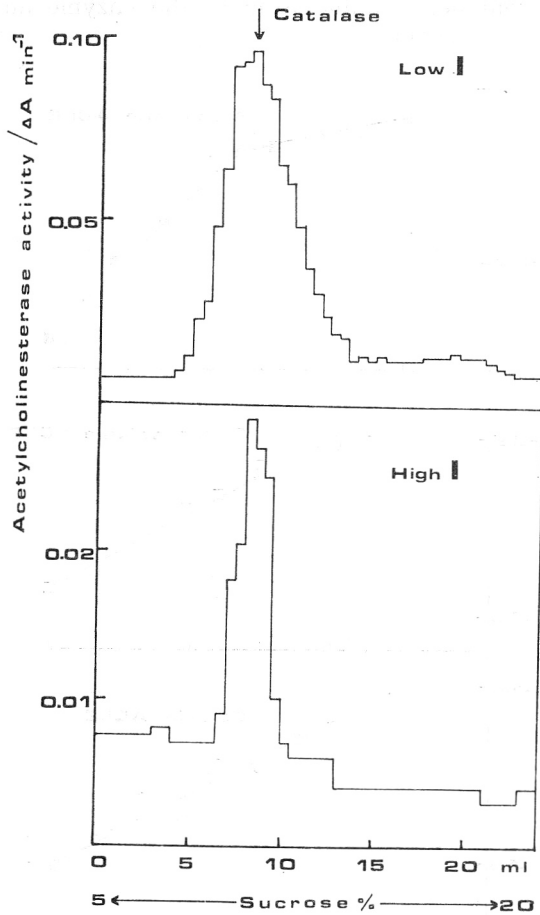

Fig. 5. Sucrose density gradient centrifugation of Triton X-100 soluble acetylcholinesterase.

Ageing of this preparation resulted in a slight loss in activity and a slight broadening of the peak but no change in the value of the sedimentation constant. Smaller peaks of higher mol. wt. were observed for the »naturally soluble« and EDTA soluble AChE and these had sedimentation constants of 15 S and 19 S approximately equivalent to mol. wt. 360 000 and 525 000. During gradient centrifugation, no molecular species less than 11.4 Sv were observed in contrast to the results obtained on electrophoresis.

Arrhenius plots

The Arrhenius plot of the membrane-bound enzyme shows two linear regions with a break at 27 °C, the transition temperature (Fig. 6a). This is often found with membrane associated enzymes and is abolished with detergent due to the loss of the membrane structure[15]. In the case of brain AChE, treatment with Triton X-100 abolishes the break and a linear plot is obtained as expected for a membrane associated enzyme (Fig. 6b). The »naturally soluble« enzyme also shows a linear plot with no break (Fig. 6c) so is probably a genuinely soluble form of the enzyme and not associated with small particles of membrane.
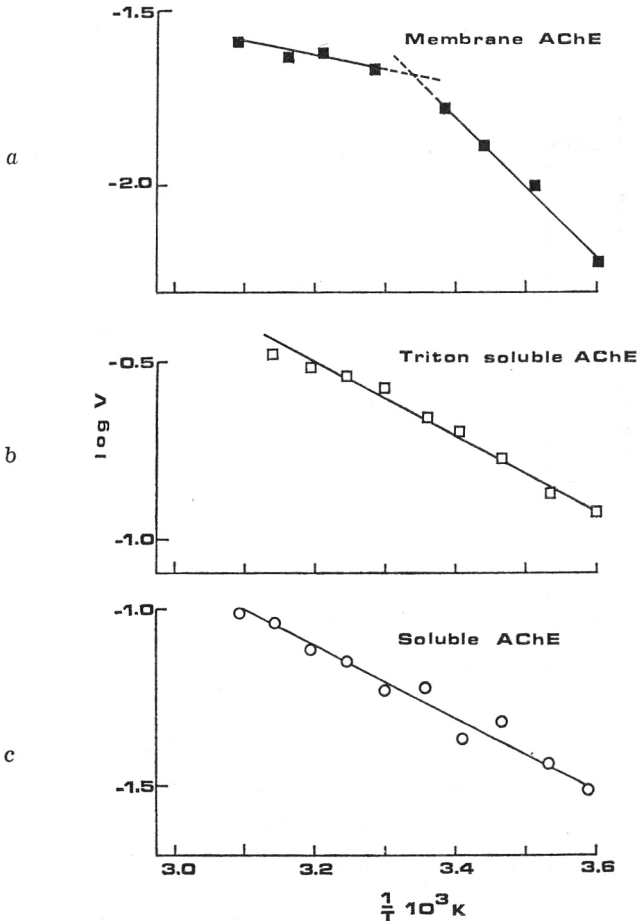
Fig. 6. Arrhenius plots of brain acetylcholinesterase. V is the maximum activity in μmol min^{-1} mg^{-1}$_{protein}$.

The energies of activation calculated from the Arrhenius plots are given in Table II. The lowest value of 8.3 kJ/mol is found in the case of the membrane enzyme above the transition temperature. Solubilization with Triton X-100 increases this value to 20 kJ/mol very similar to the value

TABLE II.

Activation energies of membrane and soluble brain acetylcholinesterase

	$E/kJ\ mol^{-1}$		
Membrane suspension	8.3	&	39
Aqueous extract	19		
Triton extract	20		
Human erythrocyte ghosts	2.2	&	22

found for the »naturally soluble« form of the enzyme of 19 kJ/mol. Below the transition temperature, the activation energy of the membrane preparation increases steeply to 39 kJ/mol.

DISCUSSION

The operational criterion of solubility used was if the AChE remained in the supernatant following centrifugation at 100 000 *g* for 1 hour. Using this standard, 15⁰/o of the total brain AChE was found to be soluble following homogenization of the brain with dilute buffer, a value similar to that reported by Bajger and Žižkovský[16].

Further solubilization was obtained after extraction of brain homogenate with 1⁰/o Triton X-100 although the yield of 65⁰/o was lower than that of other authors for the extraction of rat brain[7,17]. The combination of detergent and high salt concentration increased the amount of enzyme solubilized (Fig. 1) which could mean that both hydrophobic and electrostatic bonds are involved in binding the enzyme to the membrane. Another possibility is that the KCl weakens electrostatic bonds which bind membrane proteins that shield some of the AChE from the action of the detergent. In the case of human erythrocyte AChE there is some evidence from electron microscopy for this latter suggestion[14]. Unfortunately the method could not be used routinely because of the problems of removing lipid which salted out at KCl concentrations greater than 0.6 M. Lysolecithin was a good solubilizing agent but too expensive to be used routinely and of the other methods used only EDTA extraction proved effective although the yield of soluble enzyme was only 45⁰/o after three extractions. This method was used for some experiments since it is arguable that it is less traumatic than detergent treatment. None of the other methods investigated were examined further due to the small amount of AChE solubilized.

The pH-activity profiles and substrate velocity curves (Fig. 3) of the membrane AChE were altered only slightly on solubilization with Triton X-100 and the optimum values obtained were similar to brain AChE from other species[6,8]. The specificity of the solubilized enzyme was that a specific AChE and was identical to that shown by the »naturally soluble« enzyme suggesting a close relationship between the two preparations. Solubilization with detergent made little difference to the K_m value of the homogenate (69—80 μM) which was similar to that obtained for other species[6-8]. The »naturally soluble« enzyme however did have a lower enzyme-substrate affinity with a K_m of 220 μM.

Electrophoresis on a polyacrylamide gradient showed the presence of several molecular forms of AChE of different molecular weights. The membrane solubilized preparation with detergent showed a similar pattern to the naturally soluble enzyme with some small differences (Fig. 4). The EDTA extract had no molecular weights greater than 240 000 and had some low mol. wt. species which did not coincide with bands observed in the other preparations. The one feature in common to all three preparations was the presence of a molecular form with a molecular weight close to 250 000 a value obtained for AChE purified from electric eel.[18,19] Sedimentation of the Triton soluble enzyme on a sucrose gradient gave a single peak of 11.4 S (Fig. 5) and this species was also the dominant one in the naturally soluble and EDTA soluble enzymes although in these latter cases some higher molecular weight species were also detected of 15 S and 19 S. Purification of AChE from the electric eel often leads to a molecule with a sedimentation coefficient of about 11 S and this form appears to be a degradation product of larger molecules of 14 S and 18 S present in fresh tissue[20,21]. No species were found with mol. wt. lower than 250 000 during sedimentation studies although smaller enzyme molecules were detected after electrophoresis (Fig. 4). One possible explanation is that during electrophoresis molecules involved in aggregating the subunits are removed.

The Arrhenius plots of the membrane AChE clearly showed a distinct break at 27 °C. There are many reasons for the existence of such a break[22] but in the case of membrane bound enzymes it is likely to be due to a change in the physical state of the lipids from a mobile to a gel-like form[15]. This seems likely since treatment with detergent which destroys the membrane structure also abolished the break in the Arrhenius plot. The »naturally soluble« enzyme showed no break in the Arrhenius plot which suggests that this form of the enzyme is not associated with lipid material in the form of micromembranes but appears to be a genuinely soluble form of AChE. The activation energy of the membrane enzyme is quite low at 8.3 kJ/mol with an approximate 5-fold increase below 27 °C. The 'naturally soluble' enzyme had an activation energy of 20 kJ/mol and the Triton X-100 soluble one was very similar to this with 19 kJ/mol. Acetylcholinesterase associated with the membrane therefore appears to be a more efficient enzyme than the soluble or solubilized form due to the lower energy barrier to be overcome. The kinetic properties are however very similar as previously discussed and if the Triton X-100 soluble enzyme is similar to the membrane enzyme then the differences in the multiple molecular forms of the »naturally soluble« and Triton soluble AChE are reelatively small. The two physical forms of the enzyme appear to be similar and it is therefore possible that the two are closely related in vivo and this is under investigation.

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DISCUSSION

M. E. Eldefrawi:

Have you considered the use of white *vs.* grey matter in an attempt to distinguish axonal from synaptic enzyme?

D. T. Plummer:

No. All our studies have been carried out on the pig brain cortex. It is something to consider for future experiments.

I. Silman:

Is pseudocholinesterase usually not present in brain tissue?

D. T. Plummer:

As far as I am aware some pseudocholinesterase activity is usually found in brain and the zero activity of pig brain homogenate with butyrylcholine is surprising.

W. N. Aldridge:

In your studies on the membrane bound enzyme you obtain the low apparent energies of activation at the higher temperature range. Would you like to comment on the fact that this value is so much lower than that for the enzyme in solution. Does it have any significance for studies in which we wish to make physiological derivations?

D. T. Plummer:

The low activition energy of acetylcholinesterase in pig brain has also been observed in our laboratory for the human erythrocyte enzyme. This presumably means that the enzyme is more »efficient« when attached to the membrane compared with the soluble enzyme and the detergent solubilized acetylcholinesterase. In some ways kinetic studies on a solubilized enzyme may give a false impression of the behaviour of the enzyme *in vivo*. This is especially true if membrane material is attached to the enzyme and measurements are made on the preparation below the transition temperature.

E. Heilbronn:

I am worried about the physiological significance of all the different molecular species of acetylcholinesterase and of soluble acetylcholinesterase. The latter may simply mirror regular membrane turnover. To what extent are the former artefacts of preparation?

D. T. Plummer:

The molecular weights of the various species do appear to be related suggesting the existence of oligomers, but how they are associated with the membrane *in vivo* is something we would all like to know. Similar molecular weight species of acetylcholinesterase are found using a wide range of solubilization procedures so artefacts induced this way do not seem to occur.

P. W. Taylor:

Would it not seem more likely that the discontinuity in the Arrhenius plots represent a local structural change in the membrane rather than a true phase transition of the integral membrane? While sharp transitions are observed with artificial bilayers with homogenous phospholipid composition, transitions occurring over a broad temperature range would be the likely situation for membranes of heterogeneous composition or those containing associated proteins.

D. T. Plummer:

I agree, the discontinuity in the Arrhenius plots must represent a phase transition in the membrane in the region of the acetylcholinesterase since structural changes away from the enzyme would be expected to have little or no effect on the activity of the enzyme.

G. Hollunger:

We have found that if extraction of the brain acetylcholinesterase by water is performed in the presence of DEAE-Sephadex so that the released enzyme is immediately adsorbed almost all of it appears in the 80 000 molecular weight form. This enzyme no longer aggregates (*J. Neurochem.* **20** (1972) 82). I think that this indicates that the enzyme is released from the membrane in the low molecular weight form and that an aggregating factor is stuck to the gel when the enzyme is eluted. Could the higher molecular weight forms in such a case be some sort of artefacts?

D. T. Plummer:

I would agree that there is an enzymatically active species of M. W. about 80 000 in mammalian brain but whether this is the form in which acetylcholinesterase exists *in vivo* is not clear. The interpretation that the higher molecular weight oligomers are artefacts arising by aggregation of the 80 000 unit is of course possible, although we have found no change in the electrophoretic pattern on ageing of our preparation. Another possibility of course is that a number of oligomers exist *in vivo* and that treatment with DEAE-Sephadex removes some aggregating factor so that only the subunits are found during your method of preparation. In our laboratory we have tried using DEAE-Sephadex, but this has no effect on our electrophoretic pattern. I believe that we were not following your method exactly so this will be repeated. It must not be forgotten, however, that your work was with calf brain and our own with pig brain cortex, and this could be the reason for the differences observed.

SAŽETAK

Svojstva topljive i na membranu vezane acetilkolinesteraze prisutne u mozgu svinje

D. T. Plummer, C. A. Reavill i C. H. S. McIntosh

Približno 15% od ukupne acetilkolinesterazne (AChE) aktivnosti korteksa svinjskog mozga može se ekstrahirati razrijeđenom otopinom pufera. Upoređena su svojstva ovakvog topljivog enzima sa svojstvima enzima vezanog na membranu, koji je u topljiv oblik preveden putem ekstrakcije 1%-nom otopinom Triton X-100 ili EDTA. Oba enzimska oblika pokazuju identičnu aktivnost prema nizu iskušanih supstrata. Promjena aktivnosti s pH i koncentracijom supstrata također je slična za oba

fizička oblika acetilkolinesteraze. Sličnosti u preparacijama topljivog i detergentom otopljenog enzima, pokazane su elektroforezom na poliakrilamidnom gelu. Tri molekularne vrste s molekularnim težinama 353 000, 262 000 i 68 000 zajedničke su obim preparatima. Topljivi enzim pokazivao je također prisutnost vrste s mclekularnom težinom 135 000, a ekstrakt Tritona X-100 još i vrsta s molekularnim težinama 181 000 i 83 000.

Acetilkolinesteraza vezana na membranu pokazuje prijelom na Arrheniusovom dijagramu pri temperaturi od 27 °C, no te pojave nema nakon ekstrakcije detergentom. Topljivi enzim ne pokazuje prijelom u Arrheniusovu dijagramu, što upućuje na odsutnost tvari vezanih na membranu. Budući da ima više sličnosti nego li razlika, može se zaključiti da su ta dva fizička oblika acetilkolinesteraze međusobno vrlo usko povezana.

ODJEL ZA BIOKEMIJU,
AKADEMIJA CHELSEA, SVEUČILIŠTE LONDON,
LONDON, SW3 6LX, ENGLESKA
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*HORMONSKI LABORATORIJ
UNIVERZITETSKA KLINIKA GÖTTINGEN
HUMBOLDTALLER, ZAPADNA NJEMAČKA