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Molecular Structure and Catalytic Activity of Membrane-Bound Acetylcholinesterase from Electric Organ Tissue of the Electric Eel

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The catalytic activity of membrane-bound acetylcholinesterase in electric organ tissue was shown to be governed by diffusion--controlled substrate and hydrogen ion gradients, generated by acetylcholinesterase-catalysed hydrolysis leading to a lower substrate concentration and pH in the vicinity of the particulate enzyme.

Various solubilization procedures, including extraction with salts and detergents, chemical modification proteolysis showed that interaction of most of the acetylcholinesterase with the excitable membrane is primarily electrostatic, but that part of the enzyme seems to be more intimately associated with the membrane.

'Native' acetylcholinesterase, as isolated from fresh electric organ tissue, is a complex molecular structure in which a multisubunit head is connected to an elongated tail. Proteolytic digestion or autolysis leads to detachment of the tail and conversion of acetylcholinesterase to a globular tetramer containing four similar subunits in which each pair is connected by disulfide bonds. Further digestion leads to cleavage of the individual polypeptide chains of the subunits which are not, however, released unless the enzyme is denatured. The possible modes of attachment of the 'native' acetylcholinesterase molecule to the excitable membrane are discussed.

INTRODUCTION

Acetylcholinesterase (AChE) is widely distributed in excitable membranes of nerve and muscle and its molecular properties are of interest because of its involvement in nervous transmission[1]. A large part of the work on the molecular structure of AChE has utilized enzyme purified from the electric organ of the electric eel, *Electrophorus electricus*. Electrogenic tissue, because of its highly specialized function, is a rich source of the enzyme.

In the following, I will first describe some studies on the catalytic activity of membrane-bound electric organ AChE as compared to that of the purified, soluble enzyme. I will then go on to present some of our recent studies on the secondary, tertiary and quaternary structure of different molecular forms. Finally, I will discuss our present knowledge of the relationship of the enzyme to the membrane.

ENZYMIC ACTIVITY OF MEMBRANE-BOUND ACETYLCHOLINESTERASE

In studies on the enzymic activity of a membranous fraction enriched in AChE prepared by Karlin[2] from the electric organ of *Electrophorus electricus*, it was observed that the specific activity of the membrane-bound enzyme

(M-AChE), assayed by the hydroxamic acid method, was 2—3-fold greater than when the enzyme was assayed by following hydrolysis of acetylcholine (ACh) by measuring liberation of acid in the absence of buffer in a pH-stat. No such discrepancy was observed between the two methods when the activity of the soluble, purified enzyme (S-AChE) was determined. When the pH--dependence of enzymic activity was compared for M-AChE and S-AChE,[3,4] it was realized that the pH-dependence of M-AChE was altered by addition of buffer to the reaction mixture, whereas the activity of S-AChE was not thus affected, thus explaining the discrepancy between the two assay methods. In Fig. 1 it can be seen that S-AChE, in the presence and absence of buffer, displays a characteristic bell-shaped activity curve, as does M-AChE in the presence of buffer. However, in the absence of buffer, the pH-dependence of M-AChE has a sigmoid shape, flattening out between pH=6 and pH=8, and rising more steeply at alkaline pH values, where the activity of the soluble enzyme is decreasing.

An explanation for the anomalous activity of M-AChE was found on the basis of studies on the enzymic activity of a synthetic enzyme membrane prepared by impregnating a collodion membrane with the proteolytic enzyme papain and cross-linking the adsorbed enzyme.[5,6] In the synthetic papain-collodion membrane it had been clearly shown that analogous pH-dependence of enzymic activity on a synthetic ester substrate resulted from a lowering of the local pH in the vicinity of the enzyme, caused by generation of hydrogen ions within the membrane by enzymic hydrolysis of the substrate. It thus appears that the acetic acid released from ACh by M-AChE lowers the local pH in unstirred layers in/or around the membrane, just as was observed for the synthetic papain-collodion membrane. Addition of buffer to the medium masks this effect. It is reasonable to assume that for a given rate of reaction of M-AChE in the absence of buffer the average local pH is the same as the bulk pH at which M-AChE in the presence of buffer acts at the same rate; *e. g.* at pH=7.0 in the bulk solution, the local pH for M-AChE in the absence of buffer appears to be about pH = 5.8, and at an external pH of 9.0 the local pH is near to 6.3 (Fig. 1).

Additional evidence for involvement of local pH-gradients in controlling the activity of the membrane-bound enzyme came from the observation that solubilization of M-AChE in 1 M NaCl completely abolished the anomalous pH-dependence. Moreover, reduction of the reaction rate, by use of a poor substrate or by addition of a competitive inhibitor, should also reduce the anomalous behavior, because the lower the rate of hydrolysis the smaller should be the steady-state gradients created. This was indeed found to be the case.[3] Further investigation also showed that the apparent K_m of M-AChE was about 2-fold higher than that of S-AChE (Silman and Karlin, unpublished results), and Robaire and Kato[7] have recently reported similar findings. The increased K_m which they find for M-AChE, together with decreased substrate inhibition, can both be explained by diffusion control of local substrate concentrations.

The activity of intact isolated electroplax dissected from the organ of Sachs of *Electrophorus electricus* according to Schoffeniels and Nachmansohn,[8] can be measured under standard assay conditions by the titrimetric method.[9] When a buffer was added to the assay medium the apparent AChE activity

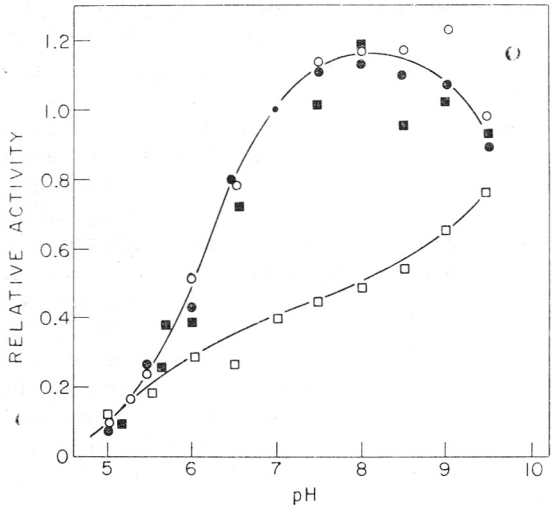

Fig. 1. pH dependence of activity of M—AChE and S—AChE in the presence and absence of buffer. Activity was measured on acetylcholine in the pH-stat. Activity in the presence of buffer was determined using a reaction mixture which contained 2mM phthalate, 1 mM phosphate and 2 mM borate. □, M—AChE in the absence of buffer; ■, M—AChE in the presence of buffer; ○, S—ChE in the absence of buffer; ●, S—AChE in the presence of buffer.

was enhanced 2—4 times (Curve A in Fig. 2) an effect apparently similar to that described above[3] for isolated membrane fragments. When the cell was then removed with forceps from the reaction vessel, little or no enzymic activity was observed in the residual medium, indicating that the enzyme remains bound to the cell. Both tryptic treatment[10] and 1 M NaCl[3] are known to remove AChE from the electroplax membrane. Addition of either of these reagents to the reaction mixture gradually led to an approximately 10-fold increase in the observed AChE activity (Curves B and C in Fig. 2). Essentially

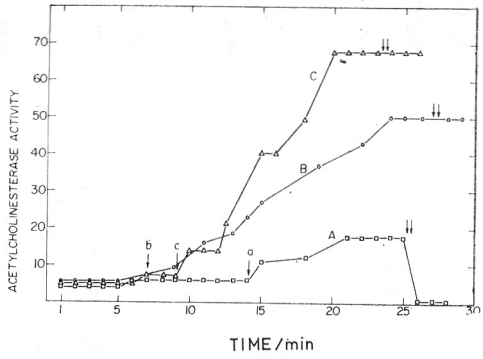

Fig. 2. The apparent AChE activity of single electroplax. Activity toward ACh, determined by the titrimetric method, is plotted vs time of incubation in different media. (A) Activity at pH = 6.8 before and after addition of 3 mM-phosphate buffer at the same pH. The buffer was added at point a. (B) Activity at pH = 7.0 before and after addition of 100 μg/ml trypsin, which was added at point b. (C) Activity at pH = 7.0 before and after changing the reaction medium from 0.1 M NaCl to 1.0 M NaCl (at point c). In all three experiments the cells were withdrawn from the reaction mixture at the point designated by a double arrow.

all the activity thus observed appeared to be solubilized, since almost no decrease in the rate of enzymic hydrolysis was observed when the cell was removed from the reaction mixture after the apparent activity had reached a plateau. Thus even in the presence of buffer, a large part of the enzyme on the intact electroplax was »latent« and its activity was not expressed unless it was detached from the cell. Latency of AChE is also demonstrated in Fig. 3 which shows the apparent activities of electric organ homogenates obtained in different homogenization media, and of the corresponding 30 000 g supernatant fluids. It should be emphasized that pretreatment with all the various reagents under the conditions employed had no effect on the activity of purified AChE. It can be seen that the highest activity was obtained when homogenization was performed at high ionic strength in the presence of the non-ionic detergent Triton X-100 or in the presence of deoxycholate at low ionic strength. Activities thus obtained were three-fold higher than those obtained on homogenization in H_2O or sucrose. Likewise 3—4 times more activity was solubilized by the detergents than by H_2O or aqueous sucrose. However, even using detergents over one third of the AChE activity remained associated with particulate structures.

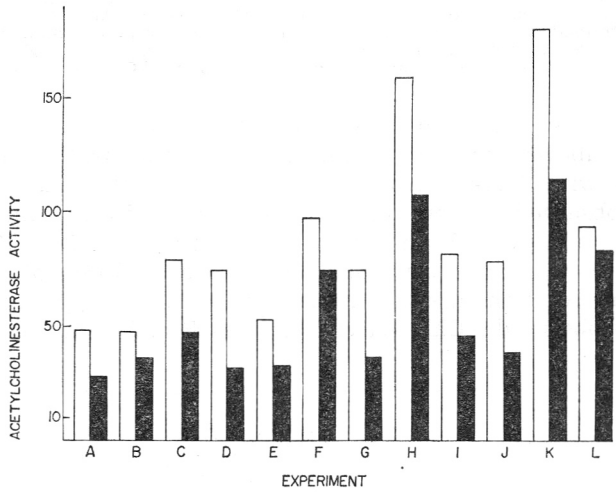

Fig. 3. AChE activity of homogenates of electric organ tissue in various homogenization media and of the 30 000 g supernatant of the homogenates. Activity was determined titrimetrically on ACh. The open columns show the activity of the homogenates and the shaded columns the activities of the corresponding 30 000 g supernatants. Homogenization was performed in the following media: A) H_2O. B) 0.33 M sucrose. C) 5 mM EDTA, pH = 7.5. D) 0.05 M Tris, pH = 7.5. E) 0.05 M NaCl — 0.05 M Tris, pH = 7.5. F) 1 M NaCl — 0.05 M Tris, pH = 7.5. G) 0.5% Triton X-100 — 0.05 M Tris, pH = 7.5. H) 0.5% Triton X-100 — 1 M NaCl — 0.05 M Tris, pH = 7.5. I) 5 mM dithiothreitol in 0.05 M Tris, pH = 7.5. J) 1 M urea in 0.05 M Tris, pH = 7.5. K) 0.5% sodium deoxycholate in 0.05 M Tris, pH = 7.5. L) 0.05 M Tris, pH = 7.5, followed by digestion with 0.6 mg/ml trypsin for 2 hours at 25 °C.

The above observations indicated that not only local pH gradients but also substrate gradients can be produced in membrane structures which may be either very invaginated, or even closed vesicular structures. Use of a substrate which is poorly hydrolysed by the enzyme should reduce diffusion control of activity as discussed above. Moreover if a lipid-soluble substrate is employed, it might be expected to penetrate through the bilayer structure of

a closed vesicle. Indophenyl acetate (IPA) is an uncharged lipid-soluble substrate of AChE which is hydrolysed very slowly relative to acetylcholine.[11] Fig. 4 shows an experiment in which equal amounts of tissue were homogenized either in 0.33 M sucrose or in 0.5% Triton X-100 + 1 M NaCl + 0.05 M Tris, pH=7.5. The relative AChE activities of the two homogenates were compared on ACh in the pH-stat in the absence of buffer, on acetylthiocholine in the presence of buffer and on IPA in the same buffer. Whereas the activity of the sucrose homogenate on ACh was only 43% of that of the Triton-NaCl homogenate, its relative activity on acetylthiocholine in the presence of Tris rose to 67% and on IPA the activity of the sucrose homogenate was 95% of that of the Triton-NaCl homogenate.

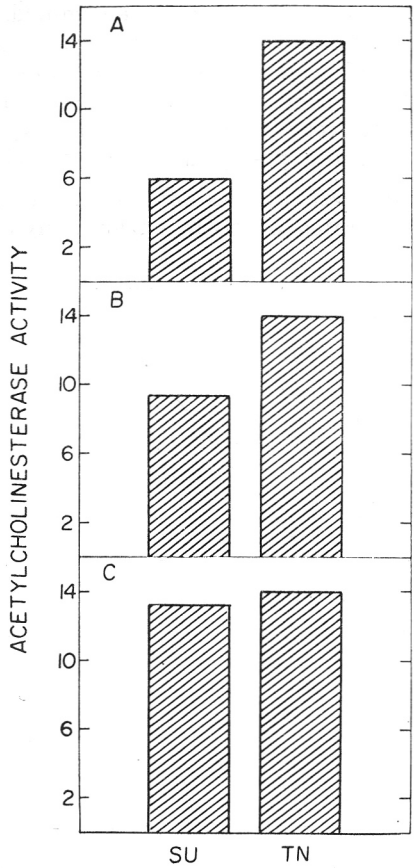

Fig. 4. AChE activity of electric organ tissue homogenates prepared either in 0.33 M sucrose (SU) or in 0.5% Triton X-100 — 1 M NaCl — 0.05 M Tris, pH = 7.5 (TN). Activity was determined on acetylcholine (A), acetylthiocholine (B) and indophenyl acetate (C).

In view of the modified enzymic behavior of particulate AChE as compared to soluble AChE, it was decided to reexamine the inhibition of AChE by anti-AChE antibodies previously reported by Williams.[12] The partial inhibition of activity on ACh observed by Williams was confirmed[13], similar inhibition being obtained for acetylthiocholine. However, a very different

picture emerged when activity towards acetylthiocholine and indophenyl acetate were compared. Fig. 5 shows an experiment in which increasing amounts of the antigen (11 S AChE) were added to a constant amount of anti-11 S AChE antiserum. Enzyme activity in the total reaction mixture was measured after incubation for 1—2 hr at room temperature. After incubation overnight at 4 °C the reaction mixtures were centrifuged and enzymic activities determined for the supernatants and for the washed and resuspended precipitates. Whereas the enzyme-antibody mixtures at different ratios showed full activity towards IPA (Fig. 5a), the activity towards acetylthiocholine in the same reaction mixtures was lower than expected from the activity of the corresponding amount of enzyme in the absence of antiserum (Fig. 5b). The difference in behavior towards the two substrates was even more marked when enzymic activity in the immune precipitates was determined. While practically no activity could be detected towards acetylthiocholine (Fig. 5b) most of the activity towards indophenyl acetate was retained. However, the activity determined in the precipitates at concentrations where all enzyme was precipitated was lower than in the total reaction mixture. This may be due to alteration in the physical properties of the precipitate after centrifugation.

It seemed that the different results obtained with the two substrates could arise from the different rates of their enzymic hydrolysis. Thus diffusion

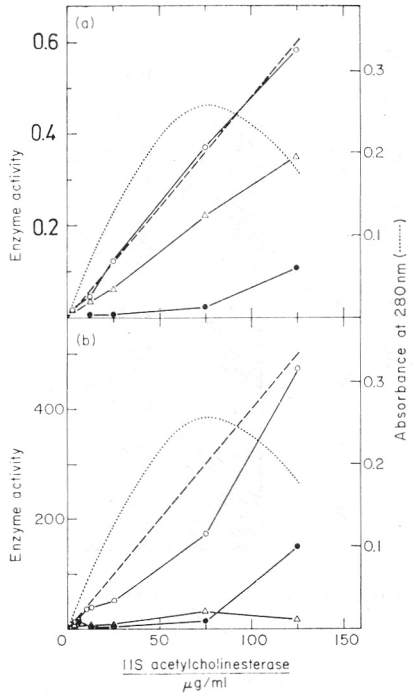

Fig. 5. Effect of anti-11S) acetylcholinesterase serum on the enzymic activity of 11S acetylcholinesterase towards indophenyl acetate and acetylthiocholine. Enzymic activities in the total reaction mixtures were determined after 1—2 h incubation, and of the supernatants and precipitates after incubation overnight at 4 °C. (○) Activity of total mixture; (●) activity in supernatant; (△) activity in precipitate; (- - -) control activity in the absence of antiserum; (. . .) precipitin curve, absorbance at 280 nm. (a) Indophenyl acetate; (b) acetylthiocholine.

control would create gradients of pH and substrate within the enzyme-antibody matrix similar to those observed for membrane-bound AChE. When the overall rate of reaction on acetylthiocholine was reduced by addition of a competitive inhibitor, as had previously been done for M-AChE (see above), the relative inhibition by antibody of AChE activity on acetylthiocholine was markedly reduced, as would be expected if diffusion control were involved. The clinching evidence for diffusion control was provided by use of the non-precipitating, monovalent F_{ab} fragment of the antibody molecule obtained by papain digestion.[14] The monovalent antibody fragments retained their antigen-binding capacity, as was shown by their ability to inhibit precipitation of AChE by intact antibodies. However, the F_{ab} preparation had no inhibitory effect on AChE activity at levels of up to 2 mg F_{ab} per reaction mixture whether activity was measured towards either acetylthiocholine or IPA.

The series of experiments described above all show that the apparent activity of particulate AChE is strongly influenced by microenvironmental effects[15] produced by the action of the enzyme itself leading to local pH and substrate gradients. These effects are particularly apparent because AChE is an especially fast hydrolytic enzyme with a turnover time of about 100 μs.[16] This high activity is probably necessary for fulfilling its biological role, rapid destruction of released acetylcholine so as to terminate impulse transmission. Whether the local pH-gradients created by membrane-bound AChE play a negative feedback role in controlling enzymic activity in the electroplax membrane or in other tissues remains an open question. However, the role of microenvironmental effects in determining apparent AChE activity should be continually borne in mind in assessing results of studies on the AChE activity of excitable tissues by both biochemical and histochemical techniques.

MOLECULAR STRUCTURE OF ACETYLCHOLINESTERASE

The physicochemical and electron microscopic studies performed by Massoulié and coworkers[17,18] and ourselves[19,20] on purified preparations of the »native« forms of AChE present in fresh electric organ tissue of the electric eel[21,22] have shown that these 'native' forms are complex molecular structures in which a multi-subunit head, apparently bearing the active-site containing polypeptides of the enzyme, is attached to an elongated tail structure of unknown composition and function. The 11 S AChE, obtained by tryptic digestion[10] or autolysis[23] is apparently a tetramer containing four similar or identical subunits.[20,24,25].

In our earlier studies we reported a molecular weight of ~ 335 000 for preparations of 11 S AChE purified from fresh or partially autolysed electric organ tissue,[20] and also presented evidence that the major polypeptide component, which contains the active site of the enzyme, has a molecular weight[19,23] of about 80 000. We also observed a minor active site-containing polypeptide of molecular weight about 60 000, as well as traces of polypeptide chains of much lower molecular weight. In our more recent studies[26] we have studied more thoroughly the polypeptide patterns of samples of AChE purified by affinity chromatography from fresh electric organ tissue subsequent to tryptic digestion, and from samples of electric organ tissue autolysed under toluene for extended periods, according to Rothenberg and Nachmansohn[27], with or without subsequent tryptic treatment. Fig. 6 shows the results obtained on

equilibrium sedimentation of three different preparations of AChE (A, B′ and C) purified from electric organ tissue after different degrees of tryptic digestion and/or autolysis. All three preparations appeared to be quite homo-

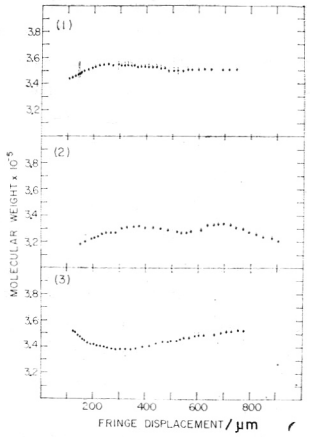

Fig. 6. Weight average molecular weight of various 11S AChE preparations, plotted against fringe displacement. 1) AChE—A: obtained subsequent to tryptic digestion of fresh electric organ tissue. 2) AChE—C: obtained from partially autolysed tissue without tryptic digestion. 3) AChE—B′: obtained by tryptic digestion of extensively autolysed tissue (stored under toluene at 4 °C for about 4 years).

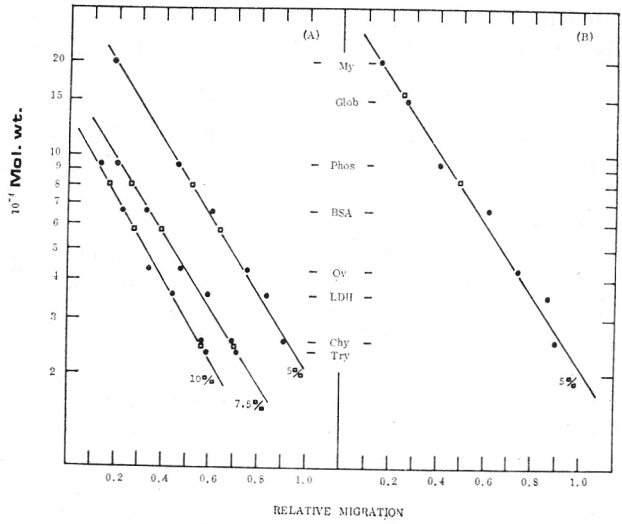

Fig. 7. Determination of the molecular weights of the principle polypeptide chains in different AChE preparations by polyacrylamide gel electrophoresis on 12 cm gels in the presence of 0.1% SDS. Electrophoresis was performed in 5%, 7.5% and 10% gels in the presence of β-mercaptoethanol (A) and in 5% gels in the absence of β-mercaptoethanol (B). No significant differences were found between the mobilities of the main polypeptide components of 11S and 'native' AChE on the gels. The marker proteins are indicated by the closed circles (●) and the AChE components by open squares (□) Each point represents the average of 3—5 experiments. Abbreviations: My-myosin, Glob — 7 S γ-globulin, Phos — phosphorylase b, BSA — bovine serum albumin, Ov — ovalbumin, LDH — lactate dehydrogenase, Chy — α-chymotrypsinogen A, Try — trypsin. The molecular weight of the ~ 25 000 polypeptide present in 11S AChE (A) was determined only for a preparation of 11S AChE purified following extensive autolysis, in which it is present in considerable amounts.

geneous, as can be deduced from the small variation of the molecular weight plotted vs. fringe displacement (which is proportional to concentration) along the ultracentrifuge cell. The preparations have molecular weights of 320 000—350 000 which are significantly higher than the molecular weights of 230 000—260 000 previously reported for preparations of 11 S AChE from *Electrophorus electricus*,[24,28,29] but resemble the value reported by Taylor *et al.*[30] for 11 S AChE from *Torpedo californica*.

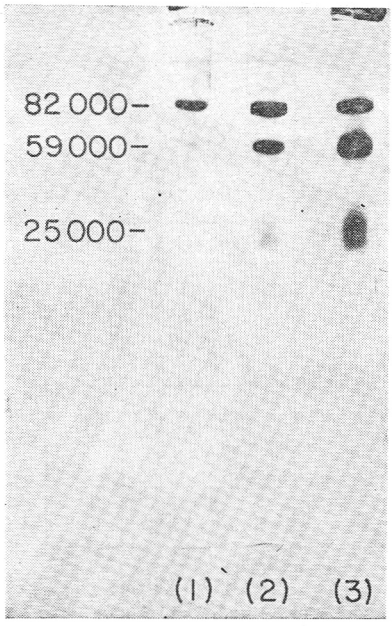

Fig. 8. Acrylamide gel electrophoresis, in the presence of SDS and β-mercaptoethanol, of AChE preparations, 5% Acrylamide gels were used and stained with Coomassie blue. 1) 'Native' AChE (14S + 18 S). 2) 11S AChE—B: obtained by tryptic digestion of partially autolysed electric organ tissue. 3) 11S AChE—B': obtained by tryptic digestion of extensively autolysed tissue.

The molecular weights of the polypeptide components of »native« (14 S + + 18 S) AChE and various 11 S samples were determined by performing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol. As can be seen from Fig. 7A the two main polypeptide components have molecular weights of 82 000 ± 6 000 and 59 000 ± ± 4 000 whether the electrophoresis is performed on 5%, 7.5% or 10% acrylamide gels. However, whereas in the 'native' forms of AChE the 82 000 polypeptide is the major component and the 59 000 polypeptide is present only in small amounts if at all (Fig. 8.1), the relative amounts of the two polypeptides vary markedly in different 11 S AChE preparations (Fig. 8.2 and 8.3). In 11 S AChE derived from tissue which had undergone the most extensive autolysis (AChE-B'; Fig. 8.3) the amount of the 82 000 component is markedly decreased and the 59 000 polypeptide becomes the dominant component, its appearance being accompanied by that of polypeptides of $\approx$ 25 000 molecular weight.

Both the 82 000 and 59 000 components observed by staining for protein contain DFP-binding sites (Fig. 9). Again, it can be seen that the relative amount of the 59 000 component increases in the extensively autolysed 11 S AChE-B′ (Fig. 9.3). No radioactivity appeared to be associated with the ~ 25 000 polypeptides, indicating that they do not contain active sites.

Polyacrylamide gel electrophoresis in the presence of SDS but in the absence of β-mercaptoethanol reveals that all the preparations of 11 S AChE display polypeptides of 168 000 ± 8 000 and 88 000 ± 4 000, both of which bind DFP (Fig. 7B, Fig. 10A). The calibration plot shown in Fig. 7B shows that even

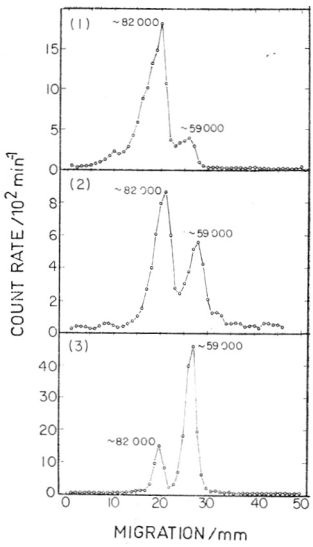

Fig. 9. Acrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol, of [³H]-DFP-labelled AChE samples. 1) Native AChE (14S + 18S). 2) 11S AChE—B. 3) 11S AChE—B.

in the absence of β-mercaptoethanol the marker proteins examined show linear dependence of log mol. wt. *vs.* electrophoretic mobility. These proteins include 7 S γ-globulin in which four polypeptide chains are linked by disulfide bonds. It thus seems plausible to assume that the 160 000—170 000 component is a dimeric structure in which two subunits are connected by intersubunit disulfide bonds.[31] This possibility was first proposed by Froede and Wilson[32] on the basis of sucrose-gradient centrifugation experiments employing ^{14}C-DFP-labelled 11 S AChE treated with either guanidine alone or with guanidine and β-mercaptoethanol.

The data presented above for the various 11 S preparations can be best explained by assuming that autolysis and/or tryptic digestion can lead to cleavage of the 82 000 polypeptide chains present in the native AChE molecule, at a limited number of susceptible sites. However, this cleavage does not lead to release of the 59 000 and ~ 25 000 fragments produced and to disruption of the quaternary structure, unless the enzyme is denatured. A similar conclusion has recently been reached by Rosenberry *et al.*[25] for AChE, and other groups have reported cases in which proteolytic cleavage per se does not change the overall mass and quaternary structure of proteins[33–36].

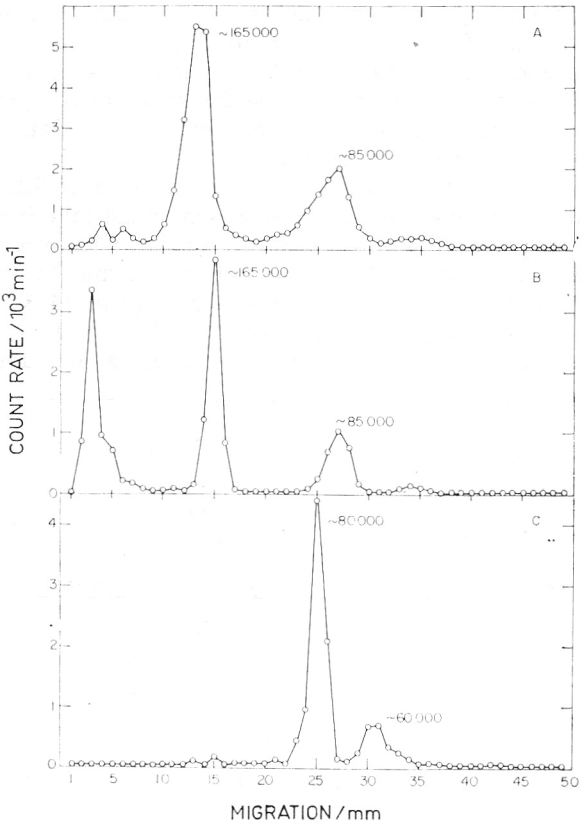

Fig. 10. SDS-polyacrylamide gel electrophoresis of [^{3}H]-DFP-labelled AChE preparations. (A) 11 S AChE, electrophoresed in the absence of β-mercaptoethanol. (B) 18 S AChE, electrophoresed as in (A). 18 S AChE (same preparation as in (B)), electrophoresed in the presence of β-mercaptoethanol.

On the basis of the above, various models can be suggested for the quaternary structure of 11 S AChE. Since »native« AChE and some 11 S preparations display relatively little 80 000 component on SDS-acrylamide gel electrophoresis in the absence of β-mercaptoethanol, it seems unlikely that a valid model will include subunits unlinked to any other subunit by disulfide bridges. We will consider, therefore, only models in which pairs of subunits are linked by disulfide bonds. Two simple models are shown in Fig. 11. In Model I the subunits are linked by disulfide bridges, as are the two parts of the polypeptide chain which are cleaved by proteolysis or autolysis. The polypeptide chains are arranged in parallel (i. e. with the same polarity from the NH_2-terminal to the COOH-terminal residue) as is the case in 7 S γ-globulin[37]. This model does not seem very satisfactory, because whatever the percentage of subunits cleaved enzymically, one would not expect appearance of 80 000 polypeptide components in the absence of β--mercaptoethanol, and this is not, in fact, the case[31] (Fig. 10A). Another simple model, model II, does not require intrasubunit disulfides, and postulates an anti-parallel arrangement of polypeptide chains linked by disulfide bridges

in each of which one cysteine residue is beyond the cleavage point. Anti-parallel arrangement of polypeptide chains in multisubunit proteins has indeed been described in several cases[38]. This model is more attractive since it can account for the appearance of an ~ 80 000 species even prior to reduction. Obviously, more complex models can account for the data assuming more disulfide bridges and/or cleavage points.

11 S AChE itself is a degradation product of 'native' AChE and the quaternary structure of the 'native' forms of the enzyme must take into account interactions between larger numbers of subunits, and also the presumably specific mode of attachment of the 'tail' of the native enzyme to the multisubunit head[18,20]. When preparations of purified »native« (*e. g.* 14 S or 18 S) AChE are electrophoresed on polyacrylamide gels in the presence of SDS and β--mercaptoethanol, the principle ~ 80 000 active-site bearing subunit is observed,

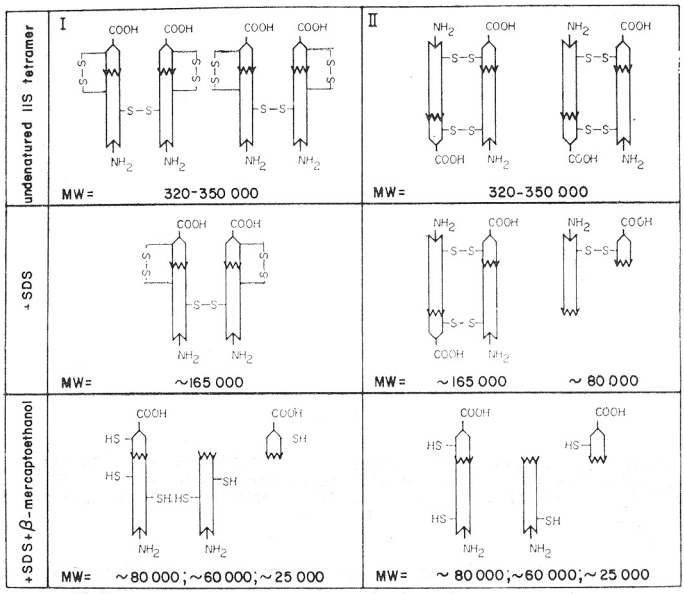

Fig. 11. Schematic models for the subunit structure of 11S AChE, together with the products to be expected under the conditions employed for SDS-acrylamide gel electrophoresis, both in the presence and absence of β-mercaptoethanol, subsequent to cleavage of either one or both polypeptides of the dimer. The site where a cleavage can occur is indicated by a serrated line.

together with traces of the ~ 60 000 component (Fig. 8.1), a pattern similar to that obtained for 11 S AChE purified from electric organ which had not been extensively autolysed[19]. No polypeptide which can be identified with the 'tail' has yet been detected, possibly because its probable low amount (~ 5—10% of the total protein)[20] is combined with low staining efficiency. However, when [^{3}H]-DFP-labelled 18 S AChE was electrophoresed in 5% acrylamide gels in the presence of SDS but without β-mercaptoethanol (Fig. 10B), about 40% of the radioactivity remained at the origin, while the remainder appeared as species of ~ 160 000 and ~ 85 000 as observed for 11 S AChE. No material remained at the origin in the presence of β-mercapto-ethanol (Fig. 10C). In order to estimate the molecular weights of the heavy components which did not enter the gel, electrophoresis of [^{3}H]-DFP-labelled

18 S AChE was also performed in the presence of SDS and in the absence of β-mercaptoethanol on 3.1% acrylamide gels. On such gels most of the heavy radioactive material was resolved into two main components of molecular weight 360 000—460 000 (Fig. 12) assuming the extrapolation of the calibration curve obtained with the polypeptide markers employed to be valid.

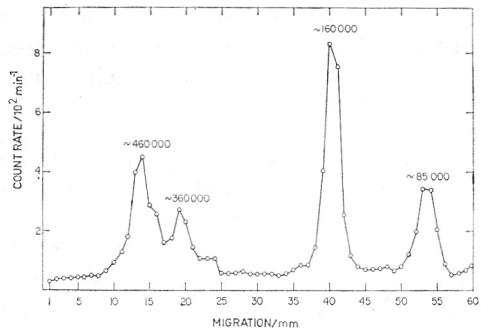

Fig. 12. SDS-polyacrylamide gel electrophoresis, on 3.1% gels, in the absence of β-mercaptoethanol, of 18 S AChE.

Experiments performed by subjecting [^{3}H]-DFP-labelled preparations of 18 S and 11 S AChE to sucrose gradient centrifugation in the presence of SDS yielded a similar pattern. The catalytic subunits were located by monitoring radioactivity on the gradients and their apparent sedimentation coefficients were determined by comparing their rates of migration with those of BSA

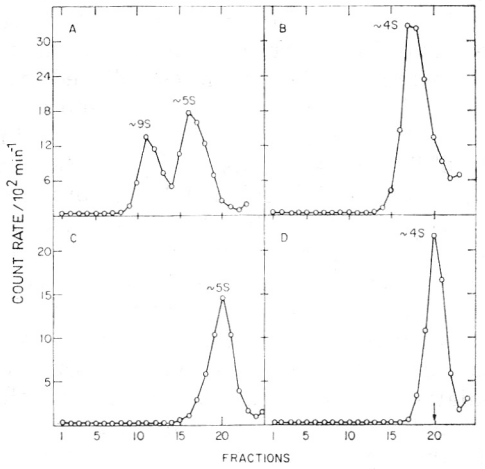

Fig. 13. Sucrose gradient centrifugation in the presence of SDS of [^{3}H]-DFP-labelled 18 S AChE (A, B) and 11S AChE (C, D). Centrifugation was performed in the presence of β-mercaptoethanol (B, D) and in its absence (A, C).

and myosin. The sedimentation coefficients of the two marker proteins in SDS were determined separately in an analytical centrifuge. It was found that 11 S AChE was dissociated into ~ 5 S particles in the presence of SDS (Fig. 13C), while in the presence of SDS and β-mercaptoethanol the enzyme was further dissociated into ~ 4 S particles (Fig. 13D). However, in the absence

of β-mercaptoethanol, 18 S AChE yielded both a 9 S species and a 5 S species (Fig. 13A), both of which yield a 4 S component on addition of reducting agent (Fig. 13B).

The similar results of the electrophoresis and centrifugation experiments in the presence of SDS and absence of β-mercaptoethanol indicate that in 'native' AChE a higher hierarchy of intersubunit bonds (or subunit-'tail' bonds) is present and that these linkages are either disulfide bonds or are masked by disulfide bonds. Thus the ~ 400 000 components revealed on the 3.1% acrylamide gels may be structures in which some of the subunits in the head have remained connected to the 'tail', either via disulfide bonds, or via non-covalent bonds which are cleaved subsequently to reduction. Alternatively, these large species may be a multisubunit structure present in 'native' AChE but absent in 11 S AChE, to which the tail is no longer attached after SDS treatment. The observation that only about 35—40% of the [^{3}H]-DFP radioactivity appears in the heavy components suggests, assuming the first possibility, that only about half or less of the subunits are directly associated with the tail, while the remainder, mainly in the form of dimers, are readily detached by SDS treatment. If the second possibility is considered, the results also suggest the presence of two different classes of subunits, one of which might also be more directly associated with the tail than the other. These observations also suggest that two types of 82 000 subunit exist, which may actually be slightly different chemically. However, as yet no additional evidence is available to support this possibility.

RELATIONSHIP OF ACETYLCHOLINESTERASE TO THE EXCITABLE MEMBRANE

Acethylcholinesterase is generally considered to be a membrane-bound protein, whether attached to excitable membranes of nerve and muscle, or to the erythrocyte membrane. In the case of electric organ tissue its association with the innervated membrane has been established by the techniques of subcellular fractionation[2], histochemistry[39] and immunofluorescence[40]. However, the precise relationship of the enzyme to the membrane in electrogenic tissue is not well understood[9,41], and attempts to apply the criteria for differentiating between integral and peripheral membrane proteins[42] yield conflicting results. The situation is complicated by the occurrence, not only in extracts of electrogenic tissue, but also of muscle[43,44] and brain[45,46] of multiple molecular species of AChE, the functional significance of which is unknown. Thus they may be associated preferentially with certain areas of the membrane[44], conceivably playing a different physiological role. Alternatively, certain molecular forms may be either degradation products of the true »native« enzyme or intermediates in its assembly. In any of these cases different forms may be associated with the excitable membrane to varying degrees. The situation is further complicated by microenvironmental effects on enzyme activity and the resulting »latency« frequently imposed on AChE activity as discussed in Section 2. Some possible modes of association of »native« AChE with the excitable membrane are shown in Fig. 14.

Our earlier experiments[3] showed that a large part of the apparent AChE activity in electric organ tissue could be solubilized at high ionic strength, indicating that the interaction of AChE with the excitable membrane was primarily electrostatic. We have also found no evidence that the enzyme so solubilised has a significant phospholipid content or tendency to bind phospho-

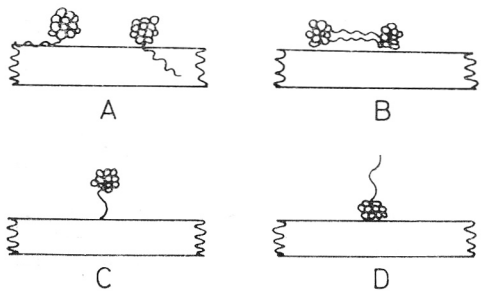

Fig. 14. Possible modes of attachment of 'native' AChE to the excitable membrane.

lipids or detergents. The observations of Massoulié and his coworkers[15,22], that the native 18 S and 14 S forms of AChE (which contain the tail structure) tend to aggregate at low ionic strength, and that their release from the membrane by proteolytic digestion is accompanied by loss of both the 'tail' and their capacity to aggregate (with concomitant transformation to the 11 S form) suggests a role for the tail in the postulated electrostatic AChE--membrane interaction (Fig. 14A or C). Our own observations that chemical modification of 'native' AChE by maleylation-which causes a change in its net charge — led to its release from the membrane in a form which had lost its capacity to aggregate at low ionic strength[9,20] also support this possibility. However, Massoulié and Rieger[22] have shown that »native« 8 S AChE, which also possesses the tail structure[18] does not aggregate at low ionic strength, although of course a small and essential part of the tail may be missing in 8 S AChE. Our own preliminary observations[47] also implicate Ca^{2+} in interaction of »native« AChE with the membrane. Thus even at low ionic strength much of the enzyme sediments together with the membrane fraction of a homogenate in the presence of Ca^{2+} but is found in the supernatant in the presence of EDTA. Even in the presence of Ca^{2+} a small amount of enzyme remains unassociated with the membrane. However, sucrose gradient centrifugation revealed that this material contained non--aggregating AChE species of sedimentation coefficient 11 S and below, while the enzyme released on removal of Ca^{2+} contained the heavier, aggregating 14 S and 18 S forms.

The tendency of »native« forms of AChE to aggregate at low ionic strength complicates interpretation of data on the distribution of AChE obtained by subcellular fractionation of electric organ tissue, in terms of the localization of the enzyme *in situ;* this is particularly so since subcellular fractionation is usually performed at low ionic strength. Such a case is illustrated in Fig. 15; which shows the AChE activity profile on a discontinuous sucrose gradient, of a membranous fraction prepared from electric organ tissue according to Changeux *et al.*[48]. According to these authors, the AChE activity peak revealed at the interface between the 0.4 M and 1.0 M sucrose layers in the gradient (Fig. 15A), corresponds to membrane-bound AChE. Indeed, electron micrographs revealed the presence of membrane fragments in the above fraction[48]. However, when »native« AChE, solubilised from electric organ tissue by treatment with 1.0 M NaCl, was separated from particulate fractions of the homogenate and then centrifuged on the same discontinuous sucrose gradient, a substantial part of the activity again accumulated at the interface between

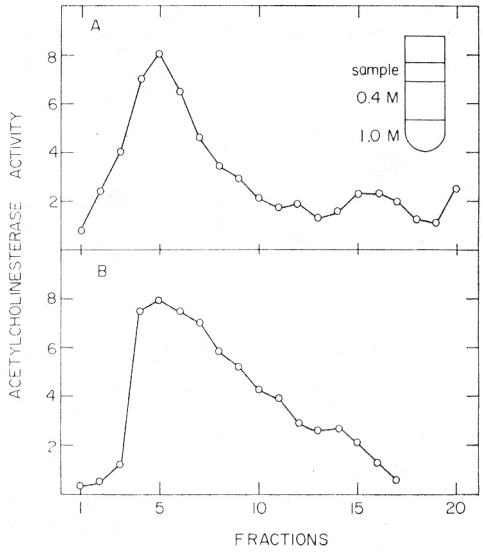

Fig. 15. AChE activity profile on a discontinuous sucrose gradient of a membranous fraction prepared from electric organ tissue according to Changeux *et al.*[48], and of AChE extracted from the tissue and separated from particulate fractions. The gradients were prepared as follows: 8 ml of 1.0 M sucrose were layered at the bottom of the tube, then 12 ml of 0.4 M sucrose, and at the top 5 ml of the sample in 0.2 M sucrose. Centrifugation was performed in a SW 25 Beckman rotor at 25 000 rpm for 5 hr, as described by the above authors. (A) Membrane fractions prepared in 0.2 M sucrose as described by Changeux *et al.*[48]. (B) AChE extracted from electric organ tissue as follows: the tissue was homogenized in 1.0 M NaCl, and the homogenate was centrifuged at 95 000 g for 70 min. An aliquot from the supernatant was dialysed overnight at 4 °C against 0.2 M sucrose and centrifuged in parallel with (A).

the 0.4 M and 1.0 M sucrose layers of the gradient (Fig. 15B). Similar experiments have also been performed with purified 'native' forms of AChE. Thus convincing evidence for AChE-membrane association demands careful histochemical and equilibrium centrifugation studies.

It was first shown by Albuquerque *et al.*[49] that treatment of skeletal muscle preparations with proteolytic enzymes destroyed functional AChE activity without, apparently, affecting the postsynaptic ACh sensitivity (*i. e.* the ACh receptor) or the presynaptic membrane. Subsequent studies by Hall and Kelly[50] using collagenase on a neuromuscular preparation and by Massoulié *et al.*[10] using trypsin on the electroplax showed that the AChE was being released into the medium in soluble form. The studies of Betz and Sakmann[51,52] established that release of AChE by either collagenase or proteases preceded what they called 'synaptic' disjunction, *e. g.* detachment of nerve terminals from the end plate. The association of the functional AChE with the synaptic membrane thus appears to be very tenuous and it is tempting to speculate that AChE may either be trapped between the basement membrane and the plasma membrane, or actually be part of the matrix of the basement membrane itself. The basement membrane which has been isolated and extensively characterized from kidney glomerulae and the lens capsule, contains collagen as a major protein constituent (for literature see Kefalides[53]). The studies of Betz and Sakmann[51,52] established that »synaptic disjunction« with either collagenase or proteases was accompanied by total digestion of the basement membrane both within the endplate and in extrajunctional areas.

If AChE is associated with the basement membrane, the possibility must be considered that the »tail« of the native enzyme is related to collagen. The elongated native AChE would then be derived from the basement membrane matrix. On sucrose gradient centrifugation of purified »native« AChE, part of the enzyme sediments to the bottom of the gradient even at high ionic strength. Electron microscopic examination of this fraction revealed the presence of dumbbell-like structures in which two multisubunit heads are connected by one or more tail fibers[20] (Fig. 16). These structures may then

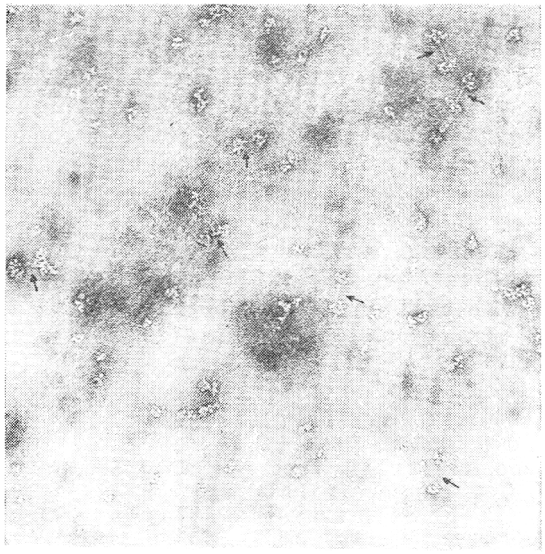

Fig. 16. Electron micrograph of a native AChE preparation containing dumbbell structures (see arrows). The field also contains multisubunit heads connected to tails and separated tails. Staining with 1% uranyl acetate. X ~ 230 000.

be closer to the native structure than the 18 S form isolated. In this connection it should be mentioned that Olsen *et al.*[54] have recently shown that elongated asymmetric structures containing collagen fibers can be isolated from basement membrane preparations. The fact that collagenase, like proteolytic enzymes, can not only release AChE from endplates[50] but can also convert »native« AChE to the 11 S form[9,20] provides supporting evidence for the collagenous nature of the tail; however, we cannot yet be sure than even the highly purified collagenase preparations we have employed are modifying AChE by collagenolytic action rather than by traces of proteolytic or esterolytic activity. We are currently attempting to provide more direct evidence for the collagenous character of the tail, and preliminary experiments (Silman, Rogozinsky and Englister, unpublished results) reveal that »native« 18 S AChE contains over 1% hydroxyproline, while 11 S AChE contains considerably smaller amounts. Hydroxyproline is found normally only in collagen and elastin, and more recently has been shown to be present in factor Clq from complement, a protein which has also been shown to contain fibrous structures associated with collagen-like sequences[55]. Its detection in »native« AChE thus provides strong support for the collagenous nature of the tail; however,

careful controls will be necessary to exclude the possibility of contamination with collagen.

All the work described above showing the ready detachment of AChE from the excitable membrane with proteolytic enzymes under conditions where the postsynaptic membrane itself is not modified yields a picture similar to that revealed by the experiments in which AChE was solubilised at high ionic strength or by maleylation. These results all indicate that AChE is a peripheral membrane enzyme or possibly even a basement membrane enzyme. However, they do not satisfactorily deal with the experiments shown in Fig. 3 which show not only that a large part of the AChE in electric organ tissue is neither active nor solubilized in 1 M NaCl, but that even in the presence of Triton X-100, the large increase in apparent activity is not accompanied by total dissociation of the enzyme from particulate matter. Some of the discrepancies may be accounted for by a combination of micro-environmental effects and actual mechanical trapping of AChE within membranous structures. However, the possibility must also be considered that there are two classes of AChE in electric organ tissue which may be attached differently to the membrane, localized differently on its surface *in situ* and conceivably fulfil different physiological roles *in vivo*. Our future research will be concerned with clarifying these issues, as well as with studying the molecular structure of »native« AChE.

REFERENCES

1. D. Nachmansohn, *Science* **168** (1970) 1059.
2. A. Karlin, *J. Cell. Biol.* **25** (1965) 159.
3. H. I. Silman and A. Karlin, *Proc. Nat. Acad. Sci. U.S.A.* **58** (1967) 1664.
4. I. Silman, *J. Gen. Physiol.* **54** (1969) 50s.
5. R. Goldman, H. I. Silman, S. R. Caplan, O. Kedem, and E. Katchalski, *Science* **150** (1965) 758.
6. R. Goldman, O. Kedem, H. I. Silman, S. R. Caplan, and E. Katchalski, *Biochemistry* **7** (1968) 486.
7. B. Robaire and G. Kato, *FEBS Lett.* **38** (1973) 83.
8. E. Schoffeniels and D. Nachmansohn, *Biochim. Biophys. Acta* **26** (1957) 1.
9. Y. Dudai and I. Silman, *J. Neurochem.* **23** (1974) 1177.
10. J. Massoulié, F. Rieger, and S. Tsuji, *Eur. J. Biochem.* **14** (1970) 430.
11. D. N. Kramer and R. M. Gamson, *Anal. Chem.* **30** (1958) 251.
12. R. M. Williams, *Proc. Nat. Acad. Sci. U.S.A.* **62** (1969) 1175.
13. D. Gurari, I. Silman, and S. Fuchs, *Eur. J. Biochem.* **43** (1974) 179.
14. R. R. Porter, *Biochem. J.* **73** (1959) 119.
15. E. Katchalski, I. Silman, and R. Goldman, *Advan. Enzymol.* **34** (1971) 445.
16. I. B. Wilson and M. A. Harrison, *J. Biol. Chem.* **236** (1961) 2292.
17. S. Bon, F. Rieger, and J. Massoulié, *Eur. J. Biochem.* **35** (1973) 372.
18. F. Rieger, S. Bon, J. Massoulié, and J. Cartaud, *Eur. J. Biochem.* **34** (1973) 539.
19. Y. Dudai, I. Silman, M. Shinitsky, and S. Blumberg, *Proc. Nat. Acad. Sci. U.S.A.* **69** (1972) 2400.
20. Y. Dudai, M. Herzberg, and I. Silman, *Proc. Nat. Acad. Sci. U.S.A.* **70** (1973) 2473.
21. M. A. Grafius and D. B. Millar, *Biochim. Biophys. Acta* **110** (1965) 540.
22. J. Massoulié and F. Rieger, *Eur. J. Biochem.* **11** (1969) 441.
23. Y. Dudai, I. Silman, N. Kalderon, and S. Blumberg, *Biochim. Biophys. Acta* **268** (1972) 138.
24. L. T. Kremzner and I. B. Wilson, *Biochemistry* **3** (1964) 1902.
25. T. L. Rosenberry, Y. T. Chen, and E. Bock, *Biochemistry* **13** (1974) 3068.

26. Y. Dudai and I. Silman, *Biochem. Biophys, Res. Commun.* **59** (1974) 117.
27. M. A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.* **168** (1947) 223.
28. W. Leuzinger, M. Goldberg, and E. Cauvin, *J. Mol. Biol.* **40** (1969) 217.
29. D. B. Millar and M. A. Grafius, *FEBS Lett.* **12** (1970) 61.
30. P. Taylor, J. W. Jones, and N. M. Jacobs, *Mol. Pharmacol.* **10** (1974) 78.
31. Y. Dudai and I. Silman, *FEBS Lett.* **16** (1971) 324.
32. H. C. Froede and I. B. Wilson, *Is. J. Med. Sci.* **6** (1970) 179.
33. A. Ullmann, F. Jacob, and J. Monod, *J. Mol. Biol.* **32** (1968) 1.
34. M. E. Goldberg, *J. Mol. Biol.* **46** (1969) 441.
35. F. Labeyrie and A. Baudras, *Eur. J. Biochem.* **25** (1972) 33.
36. C. Jacq and F. Lederer, *Eur. J. Biochem.* **25** (1972) 41.
37. W. E. Gall, B. A. Cunningham, M. J. Waxdal, W. H. Konigsberg, and G. M. Edelman, *Biochemistry* **7** (1968) 1973.
38. B. W. Matthews and S. A. Bernhard, *Annu. Rev. Biophys. Bioeng.* **2** (1973) 257.
39. F. E. Bloom and R. J. Barrnett, *J. Cell. Biol.* **29** (1966) 475.
40. P. Benda, S. Tsuji, J. Daussent, and J.-P. Changeux, *Nature (London)* **225** (1970) 1149.
41. J. B. Cohen, M. Weber, M. Huchet, and J.-P. Changeux, *FEBS Lett.* **26** (1972) 43.
42. S. J. Singer, *Annu. Rev. Biochem.* **43** (1974) 805.
43. B. W. Wilson, M. A. Mettler, and R. V. Asmundson, *J. Exp. Zool.* **172** (1969) 49.
44. Z. W. Hall, *J. Neurobiol.* **4** (1973) 343.
45. S. L. Chan, D. Y. Shirachi, B. N. Bhargava, E. Gardner, and A. J. Trevor, *J. Neurochem.* **19** (1972) 2747.
46. R. J. Wenthold, H. R. Mahler, and W. J. Moore, *J. Neurochem.* **22** (1974) 945.
47. Y. Dudai and I. Silman, *FEBS Lett.* **30** (1973) 49.
48. J.-P. Changeux, J. Gautron, M. Israël, and T. Podleski, *C. R. H. Acad. Sci., Ser. D* **269** (1969) 1788.
49. E. X. Albuquerque, M. D. Sokoll, B. Sonesson, and S. Thesleff, *Eur. J. Pharmacol.* **4** (1968) 40.
50. Z. W. Hall and R. B. Kelly, *Nature (New Biol.)* **232** (1971) 62.
51. W. Betz and B. Sakmann, *Nature (New Biol.)* **232** (1971) 94.
52. W. Betz and B. Sakmann, *J. Physiol.* **230** (1973) 673.
53. N. A. Kefalides, *Int. Rev. Conn. Tissue Res.* **6** (1973) 63.
54. B. R. Olsen, R. Alper, and N. A. Kefalides, *Eur. J. Biochem.* **38** (1973) 220.
55. K. B. M. Reid, D. M. Lowe, and R. R. Porter, *Biochem. J.* **130** (1972) 749

DISCUSSION

E. A. Barnard:

What are the number of DFP-binding sites per molecule in the dimer and in the tetramer of the native AChE?

I. Silman:

We have not yet investigated this point.

E. Heilbronn:

Is there any presynaptic AChE? Have you looked at AChE levels in denervated electric organs?

I. Silman:

We have not studied denervated electric organ tissue and do not, as yet, have any inormation about the localization of our AChE species on presynaptic, synaptic or subsynaptic membranes.

G. Hollunger:

Do you know if your 80 000 molecular weight component has any enzymatic activity? Does it aggregate to the dimer state?

I. Silman:

We have not yet obtained any enzymically active species smaller than the 11 S form, and we have not yet attempted renaturation or reassembly studies.

M. E. Eldefrawi:

In your AChE antibody experiments you found different activities of the antibody-antigen precipitate towards the two substrates. Could you eliminate activities from the precipitate entirely if you raised the antibody concentration?

I. Silman:

The maximal antibody-antigen ratio in the immune precipitate is determined by antigen valency which is a function of molecular weight, and is found at the peak of the precipitin curve, where high enzymic activity is observed towards indophenylacetate and much lower activity towards acetylthiocholine.

U. Brodbeck:

What is the S value of the dumbbell-shaped form? What are the conditions for aggregation of your purified form? What are the isoelectric points of the forms? Do you envoke 1 or 2 different subunits per tetramer?

I. Silman:

We have not accurately measured the sedimentation coefficients of the dumbbell forms of AChE, but they must be over 40 S. Aggregation of purified native AChE occurs, exactly like that of the enzyme in crude extracts, by lowering the ionic strength below approx. 0.3 M NaCl. We think that all the subunits bear active sites and are presumably homologous in sequence. However, they may possibly have different terminal sequences due to different degrees of proteolysis or autolysis. Also, possibly some may have pieces of the tail covalently attached. More detailed studies will be needed to clarify this point. We have not measured the isoelectric points of the different molecular forms. However, on free boundary electrophoresis on cellulose acetate a mixture of 14 S and 18 S AChE migrates as a single band, while 11 S AChE has approximately the same mobility but displays a very diffuse band which we attribute to microheterogeneity arising from autolysis or proteolysis.

SAŽETAK

Molekularna struktura i katalitička aktivnost na membranu vezane acetilkolinesteraze električnog organa jegulje

I. Silman i Y. Dudai

Utvrđeno je da katalitička aktivnost na membranu vezane acetilkolinesteraze električnog organa jegulje ovisi o gradijentima supstrata i vodikovih iona kontroliranih difuzijom. Niža koncentracija supstrata i pH u okolini enzima nastaje zbog hidrolize koju katalizira acetilkolinesteraza.

Različiti postupci otapanja, uključujući ekstrakciju solima ili detergentima, kemijsku modifikaciju i proteolizu, pokazali su da je interakcija većeg dijela acetilkolinesteraze s nadražljivom membranom prvenstveno elektrostatska, no čini se da je jedan dio enzima čvršće vezan na membranu.

Nativna acetilkolinesteraza, izolirana iz svježeg tkiva električnog organa, ima kompleksnu molekularnu strukturu u kojoj je »glava« s mnogostrukim osnovnim jedinicama priključena na oduljeni »rep«. Proteolitskom digestijom ili autolizom dolazi do odvajanja »repa« i konverzije acetilkolinesteraze u globularni tetramer, koji sadrži četiri slične osnovne jedinice u kojima je svaki par povezan disulfidnim vezovima. Daljnja digestija dovodi do cijepanja individualnih polipeptidnih lanaca osnovnih jedinica, koje se međutim odvajaju tek denaturacijom enzima. Raspravljene su mogućnosti načina vezivanja molekule nativne acetilkolinesteraze na nadražljivu membranu.

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