Active-Site Determinations on Forms of Mammalian Brain and Eel Acetylcholinesterase

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Three forms of brain acetylcholinesterase were purified from bovine caudate-nucleus tissue and determined by calibrated gel filtration to have mol.wts. of approx. 120 000 (C), 230 000 (B) and 330 000 (A). [^3H]Di-isopropyl phosphorofluoridate (isopropyl moiety labelled) was purified from commercial preparations and its concentration estimated by an enzyme-titration procedure. Brain acetylcholinesterase preparations and enzyme from eel electric tissue were allowed to react with [^3H]di-isopropyl phosphorofluoridate in phosphate buffer until enzyme activity was inhibited by 98 %. Excess of [^3H]di-isopropyl phosphorofluoridate that had not reacted was separated from the labelled enzyme protein by gel filtration, or by vacuum filtration or by extensive dialysis. The specificity of active-site labelling was confirmed by use of the enzyme reactivator, pyridine 2-aldoxime. The forms of brain acetylcholinesterase were calculated to contain approximately two (C) four (B) and six (A) active sites per molecule respectively. Acetylcholinesterase (mol.wt. 250 000) from electric-eel tissue was estimated to contain two active sites per molecule. Gradient-gel electrophoresis was used to confirm the estimation of molecular weights of brain acetylcholinesterase forms made by gel filtration. Under the conditions of electrophoresis acetylcholinesterase form A was stable, but form B was converted into a species of approx. 120 000 mol.wt. Similarly, form C of the brain enzyme was converted into a 60 000-mol.wt. form during electrophoresis. These results are in general accord with the suggestion that the multiple forms of brain acetylcholinesterase may be related to the aggregation of a single low-molecular-weight species.

In a previous study mammalian brain acetylcholinesterase (EC 3.1.1.7) was isolated from bovine caudate-nucleus tissue and purified to a specific activity similar to that of the pure enzyme from eel electric tissue (Chan *et al.*, 1972). The brain enzyme existed in multiple forms which were characterized in terms of molecular weight, catalytic properties, inhibitor sensitivity and glycoprotein nature (Chan *et al.*, 1972, 1973a; Gardner *et al.*, 1975). The multiplicity of forms of brain acetylcholinesterase has been noted by others (Jackson & Aprison, 1966; Hollunger & Niklasson, 1973; McIntosh & Plummer, 1973), and it has been suggested that their existence may be due to the aggregation of a low-molecular-weight form (Adamson *et al.*, 1975).

Further characterization of purified forms of brain acetylcholinesterase has included thermodynamic analyses of free energies of activation. Such analyses require accurate catalytic-site concentrations for each form and this was accomplished in the present study by labelling with radioactive di-isopropyl phosphorofluoridate. Active-site number was determined for acetylcholinesterase from eel electric tissue, both for purposes of comparison and to resolve some

controversy about the precise active-site number for the 250 000-mol. wt. form of this enzyme (Froede & Wilson, 1970; Leuzinger, 1971a).

Assessment of the molecular weights of brain acetylcholinesterase forms by gradient-gel electrophoresis revealed that they may differ in terms of their stability and that the forms may consist of multiples of a single active species with a mol.wt. of approx. 60 000. A preliminary communication has already appeared (Gordon *et al.*, 1975).

Methods and Results

Materials

General chemicals used were analytical-reagent grade. Acetylthiocholine iodide, pyridine 2-aldoxime iodide, acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; type III, electric eel) and α -chymotrypsin (type I, bovine pancreas) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Di-isopropyl phosphorofluoridate was from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and [^3H]di-isopropyl phosphorofluoridate (0.87 Ci/mmol) was from New England Nuclear Corp., Boston, MA, U.S.A. Gel-

calibration proteins were products of Boehringer Mannheim Corp., San Francisco, CA, U.S.A.

Sephadex G-10 and G-200 materials were obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A., and the collodion bags (8ml capacity) were from Schleicher and Schuell, Keene, NH, U.S.A.

Preparation of bovine brain and eel acetylcholinesterase forms

Acetylcholinesterase forms were purified from caudate-nucleus tissue of ox brains (obtained from a local slaughterhouse) by the method of Chan *et al.* (1972). The procedure involved solubilization in 0.32M-sucrose containing 1mM-EDTA then fractionation with $(\text{NH}_4)_2\text{SO}_4$. This was followed by affinity chromatography using *m*-trimethylammonium aniline as the affinity ligand and by a separation of three forms of acetylcholinesterase with different molecular weights by Sephadex G-200 chromatography. The Sephadex G-200 column had been equilibrated with and the samples were run in 30mM-Tris/HCl buffer, pH 7. The specific activities of the acetylcholinesterase forms were determined to be 450–500mmol of acetylthiocholine hydrolysed/h per mg of protein by the procedure of Ellman *et al.* (1961) and their homogeneity was verified by polyacrylamide-gel electrophoresis (Gardner, 1974). For routine experiments, the limited stability of such highly purified forms was counteracted by the addition of small quantities of the less-pure $(\text{NH}_4)_2\text{SO}_4$ precipitate of brain acetylcholinesterase to such preparations (Chan *et al.*, 1972). This introduced approx. 5% additional enzyme protein to the purified proteins, which was taken into account in subsequent calculations of active-site numbers. For determination of the active-site numbers of acetylcholinesterase derived from eel electric tissue, the commercial preparation from Sigma (type III) was used without further purification. The determination of precise concentrations of acetylcholinesterase used in labelling experiments involved comparison of observed activity (Ellman *et al.*, 1961) with the known activity of purified forms of the enzyme (Chan *et al.*, 1972). This method was accurate to within 3% when the enzyme activity of purified eel and brain acetylcholinesterase forms was correlated with protein concentration determined by the fluorescamine procedure (Bohlen *et al.*, 1973).

Purification of [^3H]di-isopropyl phosphorofluoridate

Accurate determination of the specific radioactivity of [^3H]di-isopropyl phosphorofluoridate required the removal of radioactive impurities from commercial preparations. This was done by gel filtration on columns (0.8cm $\times$ 56cm) of Sephadex G-10 and elution with distilled water (Fig. 1). Radioactive peaks I (void volume) and II lacked acetylcholinesterase inhibitory activity, whereas peak III contained over 95% of the

Fig. 1. Purification of [^3H]di-isopropyl phosphorofluoridate by gel filtration

Commercial preparations of [^3H]di-isopropyl phosphorofluoridate were chromatographed on a column (0.8cm $\times$ 56cm) of Sephadex G-10. On elution with water, fractions (0.5 ml) were collected for measurement of radioactivity and acetylcholinesterase inhibitory activity. Radioactivity peaks I in the void volume (fractions 9–12) and II (fractions 14–18) lacked enzyme inhibitory activity. Peak III (fractions 19–24) contained over 95% of acetylcholinesterase inhibitory activity in the commercial preparation and was presumed to be [^3H]di-isopropyl phosphorofluoridate.

inhibitory activity of the commercial preparations. To determine the relative homogeneity of the radioactive species in peak III, the material was incubated with a 30-fold excess of chymotrypsin (0.5 μM) for 60min at 21°C. This enzyme is a serine protease containing one site per molecule that can be phosphorylated by di-isopropyl phosphorofluoridate. On gel filtration of the incubation mixture all the radioactivity appeared in the void volume in association with enzyme protein (Fig. 2). Thus all of the radioactive material in peak III appeared to be due to a phosphorylating agent, presumably di-isopropyl phosphorofluoridate. Since it was possible that the radioactive impurities present in commercial preparations of [^3H]di-isopropyl phosphorofluoridate could be degradation products, the concentration of the parent compound was re-measured. This was done by comparison of the rates of inhibition of eel acetylcholinesterase with unlabelled di-isopropyl phosphorofluoridate (3.3 μM) and [^3H]di-isopropyl phosphorofluoridate at the same apparent concentration indicated to be present by the estimate of the manufacturer. The procedure involved the determination of bimolecular rate constants from equations of the form:

$$k_i = \frac{2.3 \log (v_1/v_2)}{(t_2 - t_1)i}$$

where v_1 and v_2 are the measured velocities of substrate reactions catalysed by the esterase remaining

Fig. 2. Binding of [^3H]di-isopropyl phosphorofluoridate to α -chymotrypsin

[^3H]Di-isopropyl phosphorofluoridate was separated from radioactive impurities present in the commercial preparation by gel filtration (see Fig. 1) and re-applied to a column (0.8 cm $\times$ 56 cm) of Sephadex G-10. On elution with water, fractions (0.5 ml) were collected for measurement of radioactivity and the [^3H]di-isopropyl phosphorofluoridate was separated as a peak of material between fractions 19–24 (□). When [^3H]di-isopropyl phosphorofluoridate was incubated with a 30-fold excess of α -chymotrypsin at 21°C for 1 h, all radioactivity appeared in the void volume (fractions 9–12) in association with enzyme protein (Δ).

uninhibited at times t_1 and t_2 of the reaction, i is the inhibitor concentration and k_i is the reaction constant (Aldridge, 1950; Hartley & Kilby, 1952). The average value of k_i from three determinations was $1.73 \times 10^{-4} \text{ M}^{-1} \cdot \text{min}^{-1}$ and $1.81 \times 10^{-4} \text{ M}^{-1} \cdot \text{min}^{-1}$ for di-isopropyl phosphorofluoridate and [^3H]di-isopropyl phosphorofluoridate respectively. Since the difference between these values represents only 4%, the quantity of [^3H]di-isopropyl phosphorofluoridate stated to be present in the commercial preparation was considered to be accurate.

Gradient-gel electrophoresis of acetylcholinesterase forms

As in our previous studies, calibrated molecular-sieve chromatography was used to estimate the approximate molecular weights of bovine brain acetylcholinesterase forms (Chan *et al.*, 1972). This method demonstrated three major acetylcholinesterase forms (A, B and C) with apparent molecular weights of 330 000, 230 000 and 120 000 respectively. Independent estimations of the molecular weights of different acetylcholinesterase forms were made by using polyacrylamide-gradient-gel electrophoresis, with calibration by marker proteins of established molecular weights (in parentheses), including aldolase (158 000), catalase (240 000), ferritin (540 000), ovalbumin (45 000) and bovine serum albumin

(67 000). These markers (10–150 μg of protein) and acetylcholinesterase samples (2–20 μg of protein) were applied to precast gradient gels (type PAA4/30, Pharmacia Fine Chemicals, Uppsala, Sweden). Electrophoresis was carried out in the Pharmacia model GE4 apparatus at 125 V (constant voltage) for 15 h in a buffer system containing 81.5 mM-boric acid, 89 mM-Tris base and 2.68 mM-disodium EDTA at pH 8.3. Control experiments established that the 15 h electrophoresis time represented equilibrium conditions. The gels were then separated into two halves, one of which was fixed in 10% (w/v) trichloroacetic acid then stained for protein with 1% (w/v) Coomassie Blue, and the other stained for acetylcholinesterase activity by the procedure of Uriel (1963).

Fig. 3 shows the electrophoretic profile of a semi-purified bovine brain and eel acetylcholinesterase preparation before the separation of forms by gel filtration (Chan *et al.*, 1972). For the brain, multiple-enzyme-staining bands were observed, three of which (A, B and C) had mol.wts. of 310 000, 250 000 and 120 000, which may correspond to the forms separated on Sephadex G-200 columns. Other acetylcholinesterase-staining bands were also present, in particular, one representing an enzyme form (D) of approx. 60 000 mol.wt. To examine if such acetylcholinesterase forms originate from the electrophoretic procedure, forms A, B and C of the brain enzyme were separated by gel filtration and then applied individually to the gradient gels for electrophoresis. As shown in Fig. 4, form A appears to be stable since the major enzyme-staining band observed on electrophoresis represents an acetylcholinesterase

Fig. 3. Gradient-gel electrophoresis of brain and eel acetylcholinesterase forms

Bovine brain and electric-eel acetylcholinesterase were obtained as detailed in the Methods and Results section and applied to gradient polyacrylamide gels (82 mm width $\times$ 76 mm length) for electrophoresis at 125 V for 15 h. The gels were then stained to reveal acetylcholinesterase activity. Band migration of the 330 000- and 60 000-mol.wt. enzyme forms was approximately 32 and 60 mm from the origin respectively. Intensity of shading corresponds to the intensity of enzyme activity.

Fig. 4. Gradient-gel electrophoresis of brain acetylcholinesterase forms separated by gel filtration

Bovine brain acetylcholinesterase was purified and separated into three forms of different molecular weight by gel filtration on Sephadex G-200 as indicated in the Methods and Results section. The forms were then applied individually to gradient polyacrylamide gels (82mm width $\times$ 76mm length) for electrophoresis at 125V for 15h. The gels were then stained to reveal acetylcholinesterase activity. For reference the migration of the 330000- and 60000-mol.wt. acetylcholinesterase forms was approx. 32 and 60mm from the origin respectively. Intensity of shading corresponds to the intensity of enzyme activity.

Fig. 5. Gradient-gel electrophoresis of combinations of brain acetylcholinesterase forms

Bovine brain acetylcholinesterase was purified and separated into three forms of different molecular weights (A, B and C) by gel filtration on Sephadex G-200 as indicated in the Methods and Results section. Combinations of forms A and C and of forms B and C were then applied to gradient polyacrylamide gels (82mm width $\times$ 76mm length) for electrophoresis at 125V for 15h. The gels were then stained to reveal acetylcholinesterase activity. For reference the migration of 330000- and 60000-mol.wt. forms was approx. 32 and 60mm from the origin respectively. Enzyme-staining bands for the combinations of forms A and C and of forms B and C are shown on the extreme right and extreme left of the Figure respectively. The three enzyme-staining bands in the centre of the Figure represent (from right to left) decreasing concentrations (0.2, 0.1 and 0.05 μ g) of acetylcholinesterase form C. Combinations of forms A and C and of forms B and C involved approx. 0.2 μ g of each component. Intensity of staining corresponds to the intensity of enzyme activity.

species with mol.wt. of 310000. However, electrophoresis of form B resulted in the appearance of an active acetylcholinesterase species of mol.wt. of only 120000 (equivalent to that of form C). The sample containing primarily form B also contained a

small quantity of form A as shown in the gradient-gel enzyme-staining profile. Similarly, subsection of purified form C to the procedure produced an enzyme species of approx. 60000mol.wt. equivalent to that of form D. The apparent lability of brain acetylcholinesterase forms B and C during gradient electrophoresis did not appear to be related to their isolation from other enzyme forms. For example, form C was observed to be converted into a smaller active species in the presence of form A (Fig. 5) and electrophoresis of a combination of forms B and C had little effect on their individual labilities. Fig. 5 also shows that the width of the enzyme-staining band representing form D appears to be a function of applied enzyme concentration and does not represent the presence of multiple forms in the mol.wt. range 50000–70000.

On Sephadex G-200 chromatography, acetylcholinesterase from eel electric tissue separated as a single, major active form with a mol.wt. of approx. 250000 (Leuzinger *et al.*, 1969; Chan *et al.*, 1973b). However, gradient-gel electrophoresis of such preparations resulted in the separation of several active species (Fig. 3) with mol.wts. estimated to be 310000 and 60000. The proportion of the lowest-molecular-weight form resolved by electrophoresis of eel acetylcholinesterase preparations was less than that observed when the brain enzyme was subjected to the same procedure.

Binding of [3 H]di-isopropyl phosphorofluoridate to acetylcholinesterase forms

The phosphorylation of acetylcholinesterase preparations was carried out in 30mM-sodium phosphate buffer, pH 8.0 at 2–4°C. Enzyme concentration was varied between 0.5 and 20 μ g in a reaction volume of 0.1 ml and the range of [3 H]di-isopropyl phosphorofluoridate concentration was from 5 to 50 μ M. Non-specific phosphorylation was minimized by monitoring enzyme activity such that the reaction was terminated by dilution with 30mM-sodium phosphate buffer when 98% inhibition of enzyme activity had occurred. The mixture was then applied to a standard Sephadex G-10 column in the same manner as that used for di-isopropyl phosphorofluoridate purification (Fig. 1) and eluted with 30mM-sodium phosphate buffer containing 200mM-NaCl at a flow rate of 4.3ml/h. The phosphorylated enzyme separated in the void volume (peak I, fractions 9–12) whereas unchanged [3 H]di-isopropyl phosphorofluoridate appeared in fractions 19–24 (peak III). Fractions 15–18 also contained radioactive material (peak II), presumed to be the phosphoric acid derivative of di-isopropyl phosphorofluoridate. Specificity of [3 H]di-isopropyl phosphorofluoridate labelling was determined by using pyridine 2-aldoxime, a reactivator of phosphorylated acetylcholinesterase, as illustrated for the eel enzyme in Table 1. The presence of 2.3 mM-

Table 1. *Effect of pyridine 2-aldoxime on [³H]di-isopropyl phosphorofluoridate labelling of acetylcholinesterase*

Eel acetylcholinesterase (2 µg of enzyme protein) was incubated with 50 µM-³H-di-isopropyl phosphorofluoridate (20000–25000 c.p.m.) in 30 mM-phosphate buffer, pH 8.0 at 2–4°C. Labelling was terminated by dilution with buffer when enzyme activity was inhibited by 98%, and the mixture was separated on a Sephadex G-10 column (see the Methods and Results section). Peak I represents enzyme protein that was eluted in the void volume. Peaks II and III represent the phosphoric acid derivative of ³H-di-isopropyl phosphorofluoridate and unchanged ³H-di-isopropyl phosphorofluoridate respectively. In some experiments pyridine 2-aldoxime (2.3 mM) was present during the incubation period or was added after phosphorylation to re-activate the enzyme. The data represent mean values for three experiments ± S.E.M.

Conditions	Total radioactivity in fractions from Sephadex G-10 chromatography (c.p.m.)		
	Peak I	Peak II	Peak III
Control	8200 ± 450	6050 ± 350	5160 ± 430
Pyridine 2-aldoxime present during incubation	400 ± 210	4800 ± 320	20000 ± 850
Pyridine 2-aldoxime added after incubation	100 ± 80	17700 ± 750	3400 ± 320

pyridine 2-aldoxime in the reaction mixture decreased radioactivity present in peak I of the Sephadex G-10 column separation to less than 5% of control values. When pyridine 2-aldoxime (2.3 mM) was added after phosphorylation, and the reaction mixture monitored for enzyme activity to the point of complete reactivation, less than 2% of the radioactivity appeared in the fraction containing enzyme protein. The increase of radioactivity in peak II, which occurred on the addition of pyridine 2-aldoxime after phosphorylation, suggests that such fractions contain phosphoric acid derivatives of ³H-di-isopropyl phosphorofluoridate. Similar experiments were carried out with different forms of brain acetylcholinesterase and in all cases ³H-di-isopropyl phosphorofluoridate labelling was greater than 95% reversible by pyridine 2-aldoxime, suggesting a minor degree of phosphorylation to non-specific sites.

Two alternative methods were used for the separation of unchanged ³H-di-isopropyl phosphorofluoridate from radioactively labelled enzyme to test the validity of the gel-filtration procedure. The first of these, collodion-bag vacuum filtration at 4°C, involves placing samples of the reaction mixture (0.1 ml) with 5 ml of the sodium phosphate buffer into 8 ml-capacity collodion bags (exclusion limit, 40000 mol.wt.). The bags were slipped into a glass tube open to the atmosphere and then inserted into a larger glass chamber attached to the laboratory vacuum line. After vacuum filtration to a volume of 0.3 ml, an additional 5 ml of the phosphate buffer was added and the procedure repeated a third time. This method removed excess of ³H-di-isopropyl phosphorofluoridate within 4 h of enzyme phosphorylation and the final volume of 0.3 ml was shown by control experiments to contain no free ³H-di-isopropyl phosphorofluoridate. The alternative procedure used was dialysis of the reaction mixture against water for 36 h. Over this time-period phosphorylated cholinesterases may be converted into forms that are not reactivated by

pyridine 2-aldoxime by the release of an alkyl or alkoxy moiety in a first-order reaction (Hobbiger, 1955; Harris *et al.*, 1966). In the case of the ³H-di-isopropylphosphorylated enzyme this 'aging' process would result in the loss of 50% of the radioactivity of the labelled molecule. To compensate for this process, the kinetics of 'aging' were determined by two methods. The first involved estimation of the degree of re-activation by pyridine 2-aldoxime as a function of time from phosphorylation, and the second method involved measurement of the loss of radioactivity from the ³H-di-isopropyl phosphorofluoridate complex with time. The results of such experiments (not shown) permitted computation of a correction factor to estimate original ³H-di-isopropyl phosphorofluoridate labelling of the enzyme protein.

Determinations of the radioactivity of samples were made in Aquasol (New England Nuclear Corp.) in a Beckman model LS 100 liquid-scintillation counter. With the preset ³H-window module, counting efficiency was 36%.

Active-site numbers for acetylcholinesterase forms

For the determination of the active-site number of eel electric-tissue acetylcholinesterase, the form that separated on Sephadex G-200 chromatography with a mol.wt. of 250000 was used. The separation of unchanged ³H-di-isopropyl phosphorofluoridate from labelled enzyme protein was carried out by column-chromatographic, vacuum-filtration or dialysis procedures. Table 2 shows that by each method, this form of eel acetylcholinesterase contained approximately two active sites per molecule of mol.wt. 250000. In the case of bovine caudate-nucleus tissue acetylcholinesterase forms, the Sephadex G-10 chromatographic method for separation of unchanged ³H-di-isopropyl phosphorofluoridate was used exclusively. Table 3 shows that brain acetylcholinesterase forms A, B and C were estimated to contain approximately six, four and two active sites per molecule respectively.

Table 2. *Active-site numbers of acetylcholinesterase from eel electric tissue*

Eel acetylcholinesterase (1–20 μg) was labelled with [^3H]di-isopropyl phosphorofluoridate under conditions described in the Methods and Results section. Labelled enzyme protein was separated from unchanged [^3H]di-isopropyl phosphorofluoridate by one of three methods: dialysis for 36 h, collodion-bag vacuum filtration or gel filtration on Sephadex G-10. Active-site numbers were calculated on the basis of a mol.wt. of the enzyme of 250 000 with appropriate corrections for non-specific phosphorylation, and also for the dialysis procedure, the process of 'aging' (see the Methods and Results section). Active-site numbers represent mean values $\pm$ S.E.M.

Separation method	No. of determinations	Enzyme protein (μg)	Active-site number
Dialysis	7	1–20	1.85 ± 0.11
Vacuum filtration	16	5–6.5	2.19 ± 0.10
Gel filtration	5	6.5	2.26 ± 0.26

Table 3. *Active-site numbers of brain acetylcholinesterase forms*

Brain acetylcholinesterase forms were separated as detailed in the Methods and Results section and labelled with [^3H]di-isopropyl phosphorofluoridate. The labelled enzyme protein was separated from unchanged [^3H]di-isopropyl phosphorofluoridate by gel filtration on Sephadex G-10. Active-site numbers were calculated on the basis of the molecular weights given, with appropriate correction for non-specific phosphorylation. The estimates of molecular weights of individual acetylcholinesterase forms were made by gel filtration (G.F.) on Sephadex G-200 and by gradient gel electrophoresis (G.E.). Active-site numbers represent mean values $\pm$ S.E.M.

Acetylcholinesterase form	No. of determinations	Molecular weight	Enzyme protein (μg)	Active-site numbers
A	4	330 000 (G.F.)	1.19	5.88 ± 0.16
		310 000 (G.E.)		5.52 ± 0.15
B	5	230 000 (G.F.)	0.6 to 7.5	3.64 ± 0.11
		250 000 (G.E.)		3.96 ± 0.12
C	4	120 000 (G.F.)	0.49 to 3.0	2.03 ± 0.12
		125 000 (G.E.)		2.11 ± 0.13

Discussion

Accurate determination of the active-site numbers of different forms of acetylcholinesterase depends on the specificity of labelling of the reactive serine moieties at the active centres of the enzyme molecule. Ideally, specific site phosphorylation should exhibit rate constants several orders of magnitude greater than non-specific site phosphorylation. Di-isopropyl phosphorofluoridate interacts with acetylcholinesterase more slowly than certain other phosphorylating agents, which is due in part to the absence of a quaternary nitrogen atom capable of association with anionic components of the active site. Therefore, to lessen non-specific site phosphorylation, the excessive exposure of acetylcholinesterase preparations to [^3H]di-isopropyl phosphorofluoridate was avoided by terminating the reaction at the point of 98% inhibition of enzyme activity. The specificity of active-site labelling with [^3H]di-isopropyl phosphorofluoridate was confirmed by its reversibility with pyridine 2-aldoxime which interacts with anionic sites to provide appropriate orientation of the oxime moiety relative to the phosphorylated serine residue of acetylcholinesterase (Wilson & Ginsburg, 1955). With such controls and by using Sephadex G-10 chromato-

graphy to remove excess of [^3H]di-isopropyl phosphorofluoridate active-site numbers could be determined on acetylcholinesterase samples containing as little as 0.5 μg of enzyme.

Though acetylcholinesterase from mammalian brain has been investigated less thoroughly than the enzyme from eel electric tissue, most studies have demonstrated a multiplicity of forms that differ in molecular weights (Jackson & Aprison, 1966; Chan *et al.*, 1972; Hollunger & Niklasson, 1973; Adamson *et al.*, 1975). It has been suggested that different forms of brain acetylcholinesterase may all be related to a single low-molecular-weight form via aggregation processes (Adamson *et al.*, 1975) and some evidence exists that such processes may be reversible (Viana, 1974). In the present study, gel electrophoresis of bovine acetylcholinesterase preparations revealed the presence of an active form of the enzyme with an approximate mol.wt. of 60 000. This species (form D) is similar in molecular weight to the smallest active form of pig brain acetylcholinesterase (McIntosh & Plummer, 1973) and slightly smaller than a low-molecular-weight species from mouse brain (Adamson *et al.*, 1975). Acetylcholinesterase form D appears to originate in part from the form of

120000 mol.wt. (form C) which suggests the possibility of disaggregation processes occurring during electrophoresis. Similarly, electrophoresis of form B of the bovine enzyme results in its conversion into a species of approx. 120000 mol.wt., but in this case the latter form accumulates and is not converted into a smaller active form of acetylcholinesterase. Such accumulation does not appear to be due to the presence of form B, as shown by the results in Fig. 5. Thus acetylcholinesterase forms of apparently similar molecular weights can exist, but with varying susceptibility to conversion into smaller active species on electrophoresis. Whether this represents differences in subunit composition or in macromolecular assembly characteristics of the two 120000-mol.wt. forms remains to be determined. Form A of bovine brain acetylcholinesterase (mol.wt. 320000) appears to be a relatively stable form of the enzyme, since it was not converted into smaller species under the conditions of electrophoresis.

Since the estimated molecular weights of larger bovine brain acetylcholinesterase forms represent approximate multiples of the 60000-mol.wt. species, it is natural to suggest that the latter form represents the basic unit of acetylcholinesterase oligomers. However, the determination of two active sites per molecule of the 120000-mol.wt. form (C) of acetylcholinesterase has two possible interpretations. One is that form C consists of two 60000-mol.wt. species each of which contains a single active site. Clearly, the alternative interpretation is that both active sites are present on a single molecule of the 60000-mol.wt. species, the catalytic unit, which may interact with an inactive unit of virtually the same molecular weight. These two possibilities are not distinguishable on the basis of the present data, which are, however, in general agreement with the suggestion that multiple forms of brain acetylcholinesterase may be related to aggregation processes. Thus the observed values of active-site numbers on forms B and A of brain acetylcholinesterase were as predicted from associations of two and three molecules of the 120000-mol.wt. species respectively.

For eel electric-tissue acetylcholinesterase, the number of active sites present has been the subject of discussion. For eel acetylcholinesterase of mol.wt. between 224000 and 260000, active-site numbers have been reported as 2 (Leuzinger, 1971a,b), 3.3 (Rosenberry *et al.*, 1972), 4 (Froede & Wilson, 1970) and 6 (Millar & Grafius, 1970). The present study, using direct labelling with [^3H]di-isopropyl phosphorofluoridate, revealed two active sites on the eel acetylcholinesterase form of 250000 mol.wt. This value confirms the estimate of Leuzinger (1971b), who used indirect inhibitor titration methods. Gradient-gel electrophoresis of eel acetylcholinesterase preparations demonstrated an active species of approx. mol.wt. 60000. It is possible therefore that the 250000-

mol.wt. form of the eel enzyme consists of two active units and two inactive units of similar or slightly greater molecular weight (Leuzinger, 1971a).

It is of some interest that the eel and brain acetylcholinesterase forms of similar mol.wt. (230000–250000) contain two and four active sites per molecule respectively. Highly purified forms of eel and brain acetylcholinesterase exhibit quite similar specific activity values when determined by the thiocholine colorimetric procedure (Chan *et al.*, 1972). Consequently, this discrepancy in active-site numbers raises the possibility of fundamental catalytic differences between acetylcholinesterase forms as a function of species origin.

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