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ACETYLCHOLINESTERASE FROM THE HOUSE-FLY HEAD

MOLECULAR PROPERTIES OF SOLUBLE FORMS

R.W. STEELE and B.N. SMALLMAN

Dept. of Biology, Queen's University, Kingston, Ontario (Canada)

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Summary

1. Polyacrylamide gel electrophoresis in Tris/glycine buffer (pH 8.3) revealed five forms of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) in the 100 000 × *g*, 1-h supernatants of aqueous fly-head extracts from the DDT/S strain. Five other housefly strains (CSMA, Bayer 21/199, Cradson/P, Malathion/R and DDT/R) were shown qualitatively to have the same soluble forms of the enzyme.

2. Plots of the electrophoretic mobility versus polyacrylamide concentration indicated that the multiple forms constituted a size isomer family. From the retardation coefficients derived from these plots, molecular weight estimates were obtained; these suggested that the smallest active component was a form of approx. 80 000 daltons. The higher aggregates, however, did not appear as simple oligomers of this component.

3. Density gradient sedimentation supported the electrophoretic findings. The smallest active component, with a sedimentation coefficient of 5.3 S, was confirmed as a molecular species of acetylcholinesterase that has not previously been obtained from house-flies; higher aggregates gave sedimentation coefficients of 7.4, 7.8, 8.1, and 11.8 S.

4. Gel-filtration on calibrated Sephadex G-150 columns provided further evidence that the smallest active component was a form of about 80 000 daltons.

5. Autolysis converted much of the particulate enzyme and all of the soluble forms into a species of approx. 160 000 daltons indistinguishable from the native 7.4-S form. Both the autolysed enzyme and the native 7.4-S form were susceptible to cleavage by disulphide reducing agents, and released catalytically active subunits that corresponded to the 5.3-S form of 80 000 daltons. The data were compatible with a monomer-dimer relationship between the 5.3-S and 7.4-S forms.

6. The possibility is suggested that a form of molecular weight approx. 80 000 constitutes the "fundamental unit" of insect cholinesterase.

Introduction

While there has long been interest in the kinetic properties of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from insect sources, only recent studies have made progress in the resolution and characterization of the molecular properties of multiple soluble forms of this enzyme. Krysan and Kruckeberg [1] demonstrated sedimentation interconvertibility of acetylcholinesterase forms from the mayfly and honeybee. From each of these species they defined at least four forms, all of which were converted to a 7.3-S form following dialysis, dilution, or pH change. For instance, in their system heavier forms isolated at pH 6.8 were stated to be instantaneously "deaggregated" to the 7.3-S form at pH 8.0. These results conformed with earlier findings on the acetylcholinesterase of house-fly heads [2,3], which had established by gel filtration and sucrose gradient sedimentation that the aggregates of this enzyme were also reducible to a 7.3-S form (molecular weight approx. 160 000). Krysan and Kruckeberg [1] therefore proposed that a 7.3-S form was a "fundamental unit" of insect acetylcholinesterase.

In apparent accord with their observations of spontaneous deaggregation, Krysan and Kruckeberg [1] found only a single band of acetylcholinesterase activity when their soluble mayfly preparations were examined by polyacrylamide gel electrophoresis at pH 8.0. However, this result is difficult to reconcile with the multiple forms of insect acetylcholinesterase detected electrophoretically at a similar pH by other workers [4—8]. Especially significant in this context was the electrophoretic demonstration at pH 8.3 of four soluble house-fly head 'isozymes' by Eldefrawi et al. [8], and a further three distinctly different soluble 'isozymes' from the house-fly thorax [9]. When kinetically characterized by a gel-scanning technique [10], all seven 'isozymes' of house-fly acetylcholinesterase were found to differ profoundly in reactivity towards acetylthiocholine as substrate, and to a variety of organophosphate inhibitors [9,11]. In vivo inhibition studies led Tripathi and O'Brien [9] to suggest that the intrinsic reactivity differences implied a physiological role for these multiple forms. However, in view of alterable sedimentation characteristics, Krysan and Kruckeberg [1] saw no reason to regard higher molecular weight aggregates as being of any real structural or functional significance.

These ambiguities about the molecular basis of multiple soluble forms of insect acetylcholinesterase prompted us to re-examine the idea of a 'fundamental unit' and its relation to higher forms. Our results with acetylcholinesterase from the house-fly head provide further evidence of aggregate convertibility, and indicate that the 7.3-S form is in fact the homologous dimer of an active 5.3-S form of about 80 000 daltons.

Materials and Methods

Materials

The DDT/S strain of *Musca domestica* L., source Canadian Dept. of Agriculture, London, Ontario, was used routinely for investigation. Comparison was also made with other selected strains: these were the CSMA strain, source Canadian Dept. of Agriculture, Ottawa, Ontario; the Bayer 21/199, Cradson/P, Ma-

lathion/R, and DDT/R strains (source U.S. Dept. of Agriculture, Gainesville, Fla.). Each strain was reared by the method of Fisher and Jursic [12].

Bovine serum albumin and ferritin were purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Catalase, ovalbumin, α-chymotrypsin, myoglobin, and pepsin were obtained from Sigma Chemical Company, St. Louis, Missouri. Sephadex G-150 and Blue Dextran 2000 were products of Pharmacia Fine Chemicals Inc., Dorval, Quebec. All other chemicals were of the highest purity available.

Enzyme preparation. Heads were collected from unsexed 3—4 day old houseflies by the method of Moorefield [13], carefully freed of other components, and stored at −20°C until further use. The frozen heads were homogenized (10% w/v) in ice-cold distilled water using a Tekmar SDT 182N tissumizer for 20 s at 20 000 rev./min. Homogenates were centrifuged at 100 000 × *g* for 60 min at 4°C, and the supernatants were used as source of soluble enzyme.

Assay methods. Acetylcholinesterase activity was measured on a Cary Model 14 spectrophotometer by the colorimetric method of Ellman et al. [14], using acetylthiocholine iodide (0.75 mM) as substrate. As a complementary technique, the gel-scanning method of Chiu et al. [10] was employed to estimate the relative activities of acetylcholinesterase forms separated by polyacrylamide gel electrophoresis.

Catalase activity was determined by the dichromate/acetic acid assay of Sinha [15].

Electrophoresis. Polyacrylamide-gel electrophoresis was carried out in Tris/glycine (pH 8.3) essentially as described by Davis [16]. Gels were polymerized in glass tubes 9 cm long × 4 mm internal diameter, after rinsing in Kodak Photo-Flo 200 solution (0.5% v/v), and air-dried before use. Both sample and spacer gels were omitted unless otherwise stated, the samples being layered directly on top of the separation gels.

Samples were routinely made up to 10% w/v sucrose, and electrophoresis was performed at room temperature with runs initiated and maintained at 200 V (approx. 30 V/cm gel). The bromophenol blue dye front was allowed to migrate 6.5 cm before electrophoresis was terminated. When required, the dye migration front was marked by insertion of a fine stainless steel wire, after the procedure of Hedrick and Smith [17].

The direct-coloring thiocholine method [18] was used to localize acetylcholinesterase activity in gels. Each gel was incubated with intermittent stirring at 25°C in 50 ml of staining solution (0.75 mM acetylthiocholine iodide; 0.1 M phosphate buffer, pH 6.0). After satisfactory visualization, the staining reaction was terminated by direct transfer into the storage solution of 7.5% v/v acetic acid. Quantification of activity was estimated from peak absorbance at 620 nm after the gel-scanning method of Chiu et al. [10]. Densitometric scans were made on a Joyce-Loebl Chromoscan, slit 0.2 × 1.0 mm.

Sucrose gradient centrifugation. Sedimentation behaviour of fly-head acetylcholinesterase was determined as described by Martin and Ames [19]. Linear sucrose gradients (5—20% w/v; volume 4.6 ml) buffered with 50 mM Tris · HCl (pH 7.5), were carefully overlayered with 0.1 ml of enzyme preparation containing 25 μg of catalase as internal standard. Sedimentation was performed by centrifugation at 4°C in a Spinco SW-39 rotor at 38 000 rev./min. Ten drop

fractions (approx. 150 μl) were collected from a puncture hole in the bottom of each tube.

Each centrifugation run consisted of three replicate gradients. Gradient 1 was used for spectrophotometric assay; 100 μl samples of each fraction were assayed for acetylcholinesterase activity by the Ellman technique [14], and 20 μl samples were used to assay catalase activity [15]. Gradient 2 was subjected to polyacrylamide gel analysis in order to establish the identity of the molecular forms contributing to the acetylcholinesterase activity obtained in gradient 1. The resolution of all multiple forms of acetylcholinesterase required the electrophoresis of 50 μl samples at both 5% and 10% polyacrylamide concentration. Quantitative results were estimated by the gel-scanning method after Chiu et al. [10]. Catalase sedimentation was determined as previously. Gradient 3 was used as a back-up replicate, and samples from specific fractions of interest were adjusted and assayed as desired to improve resolution.

Sephadex G-150 chromatography. Gel filtration studies were performed at 4°C by ascending elution on Sephadex G-150 columns calibrated [20] with Blue Dextran 2000, bovine serum albumin (dimer 134 000; monomer 67 000), ovalbumin (45 000), α-chymotrypsin (25 000), and myoglobin (17 800). Protein elution was monitored at 280 nm using a Chromatonix Model 220 monitor (flow cell 50 μl), and acetylcholinesterase activity was assayed by both the Ellman spectrophotometric method [14] and the gel-scanning technique [10].

Results and Discussion

Validation of five multiple forms

Fig. 1 shows activity patterns obtained with an aqueous extract of fly-heads from the DDT/S strain. Five bands of activity were clearly resolved with 4—10% polyacrylamide gels; that these bands represented specific acetylcholinesterase activities was established by substrate and inhibition chracterization [8] with butyrylthiocholine iodide (0.75 mM) and eserine (1 μM), respectively. These five forms of fly-head acetylcholinesterase are hereafter referred to as acetylcholinesterases 1—5 in order of decreasing mobility [21]. It should be noted that Tripathi et al. [9,11] numbered their acetylcholinesterase 'isozymes' in the reverse order.

Our results differ from the findings [9,11] of these authors in that they found only four acetylcholinesterase forms in aqueous head extracts of the Wilson susceptible strain, whereas with the DDT/S strain we found five forms. The question arises whether the additional acetylcholinesterase form demonstrated with the DDT/S strain represents an acetylcholinesterase isoenzyme absent from the Wilson susceptible strain. If DDT/S flies possess a strain-specific form of acetylcholinesterase, then comparison with a number of other strains would be expected to reveal this difference.

Fig. 2 shows 10% polyacrylamide gels of aqueous head extracts from housefly strains with a variety of insecticide-selection histories. Without exception, five acetylcholinesterase forms were observed, though the slowest migrating form (acetylcholinesterase 5) failed to penetrate the 10% gels shown. When run at lower gel concentrations, as in Fig. 1, this form was invariably demonstrated. In every instance the mobilities of forms relative to the marker dye (R_F), cor-

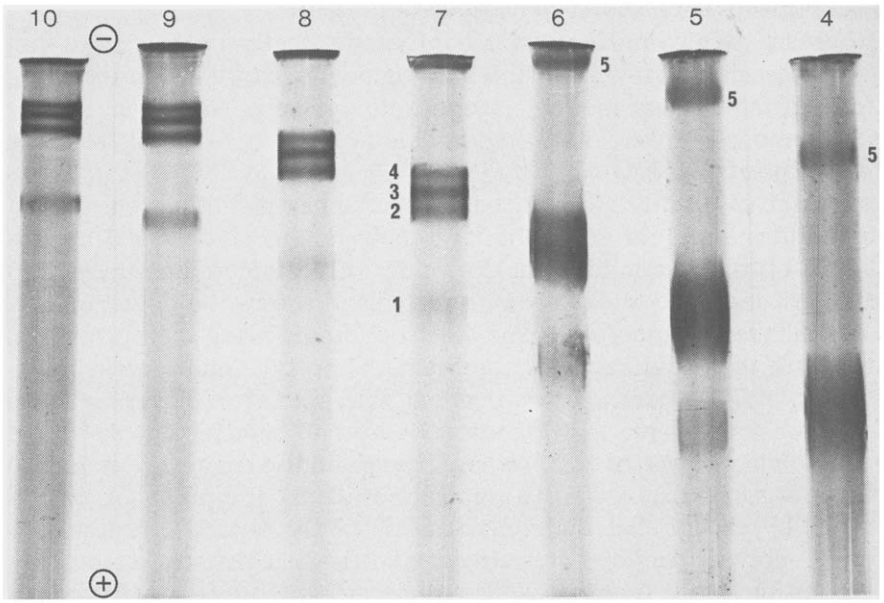

Fig. 1. Polyacrylamide gel electrophoresis in Tris/glycine buffer (pH 8.3) of a 10% w/v aqueous soluble fly-head extract (100 000 × *g*, 1-h supernatant) form the DDT/S strain. Samples (50 µl/gel) containing 0.5 I.U./ml acetylcholinesterase activity were run and stained as described in Materials and Methods. Gels of 10—4% polyacrylamide concentration are illustrated in order to demonstrate acetylcholinesterases 1—5.

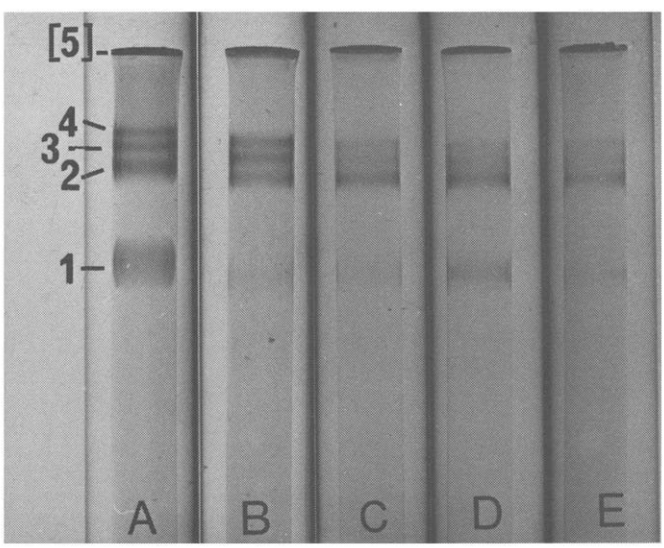

Fig. 2. Polyacrylamide gel electrophoresis of aqueous soluble fly-head extracts (10% w/v) from house-fly strains with various insecticide-selection histories. Gels of 10% polyacrylamide concentration which were stained for acetylcholinesterase activity are illustrated: (A) CSMA susceptible strain; (B) Bayer 21/199 resistant strain; (C) Cradson/P multi-resistant strain; (D) Malathion/R resistant strain; and (E) DDT/R resistant strain.

responded to respective R_F values of acetylcholinesterases 1—5 from the DDT/S strain. Moreover, this coincidence was confirmed directly by co-electrophoresing extracts of each strain with DDT/S material, a procedure which would be expected to discriminate any microelectrophoretic variance. No variances were detected and zymograms were indistinguishable in qualitative detail from the DDT/S zymograms of Fig. 1.

We next sought to establish where the additional acetylcholinesterase form fits into the sequence of four acetylcholinesterase 'isozymes' observed by Tripathi et al. [9,11], by determining the R_F values of acetylcholinesterases 1—5 under conditions described by these authors. Fig. 3 shows the densitometric scan of a DDT/S zymogram prepared and electrophoresed using their protocols. In agreement with their findings, only four hands of acetylcholinesterase activity were resolved in 7% separation gels overlaid with 4% (C = 20%) spacer gels. The possibility was considered that the lower number of bands might be caused by a poorer resolving power of this system. Therefore, the enzyme preparation used for Fig. 3 was again subjected to electrophoresis by the protocols of Tripathi et al. [9,11], except that the concentration of the separation gel was increased to 10% polyacrylamide. This single modification resulted in our unequivocal resolution of all five forms. This observation, coupled with the previous demonstration of acetylcholines 1—5 in a variety of selected strains (Fig. 2), provides substantial evidence that five multiple forms of acetylcholinesterase are characteristic of aqueous soluble extracts of house-fly heads.

Molecular characterization of the five multiple forms

(a) Ferguson plots. The Ferguson relationship ($\log M = \log M_0 - K_R T$, where M = the electrophoretic mobility and is related to R_F; M_0 = the free electrophoretic mobility; K_R = the retardation coefficient; and T = the total gel concentra-

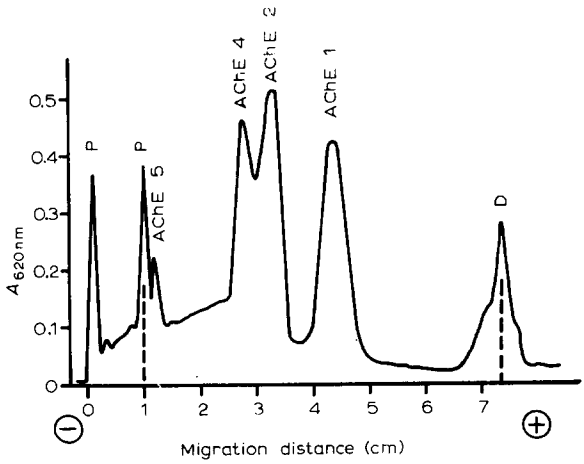

Fig. 3. Densitometric scan at 620 nm of acetylcholinesterase (AChE) activity in an aqueous soluble extract, prepared and electrophoresed on 7% polyacrylamide gels at 4° C after the procedures of Tripathi et al. [9,11]. Optical dichotomies at the boundaries of the 1 cm spacer gel (4% T; C = 20%) gave rise to the sharp peaks designated P on the densitometric curve; the diffuse peak D at the bromophenol blue migration front represents pigmented material characteristically found in crude fly-head preparations.

tion (acrylamide monomer + crosslinking agent), [22]), provides a useful method of protein physicochemical characterization [17,23].

Fig. 4 shows the Ferguson plots of acetylcholinesterases 1—5, derived by the weighted least-squares regression of Rodbard and Chrambach [24] from R_F measurements of a replicate series (n = 4) of 4—10% polyacrylamide gels. Extrapolation of these plots gave a family of non-parallel lines intersecting near 2% polyacrylamide concentration. By the definition of Hedrick and Smith [17], acetylcholinesterases 1—5 thus satisfy the criterion of a size isomer family of proteins.

Molecular weights of the five forms were then estimated using the derived retardation coefficients (K_R) as described by Rodbard and Chrambach [24]. A standard curve of geometric mean radius ($\bar{R}$) against $K_R^{1/2}$ was prepared for pepsin, bovine serum albumin (monomer, dimer, trimer, and tetramer), catalase, and ferritin. The $K_R^{1/2}$ values of acetylcholinesterases 1—5 were fitted to this curve and the corresponding $\bar{R}$ values were converted into molecular weights. The molecular weights thus obtained were: acetylcholinesterase 1, 84 000; acetylcholinesterase 2, 149 000; acetylcholinesterase 3, 174 000; acetylcholinesterase 4, 184 000; and acetylcholinesterase 5, 650 000. These estimates indicate that the acetylcholinesterase 1—5 size family do not constitute a simple oligomeric series. Moreover, they provide the first evidence that acetyl-

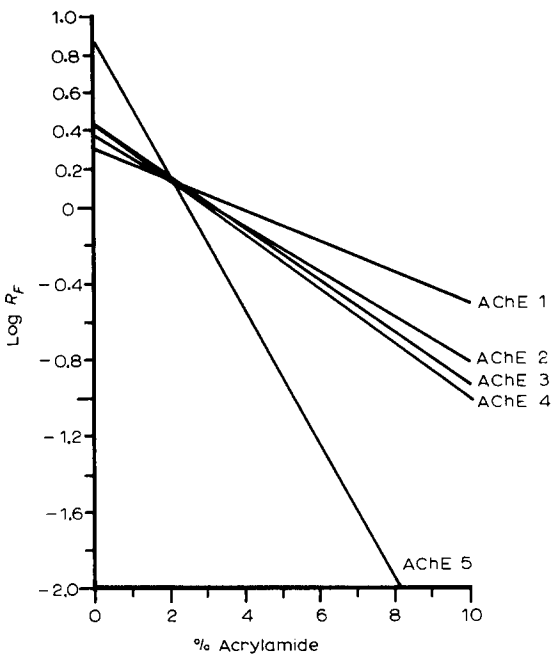

Fig. 4. Ferguson plots of acetylcholinesterases 1—5 of the DDT/S strain derived by the weighted least-squares regression of Rodbard and Chrambach [24]. R_F measurements were taken from densitometric curves of a replicated series (n = 4) of 4—10% polyacrylamide gels stained for acetylcholinesterase (AChE) activity; bromophenol blue migration fronts were marked by the procedure of Hedrick and Smith [17]. Deviations from the regression lines were insignificant and for purposes of clarity these lines are presented without their experimental data.

cholinesterase 1 is a smaller molecular species than the presumed 'fundamental unit' of 160 000 postulated by Krysan and Kruckeberg [1].

(b) Sucrose gradient centrifugation. In an attempt to verify these molecular weights, the enzyme was subjected to density gradient centrifugation for 10 h at 38 000 rev./min, essentially the same conditions used by Krysan and Chadwick [2]. In excellent agreement with their findings only two smooth peaks of activity were discriminated by the Ellman assay [14]. The heavier peak was found by analysis on 5% polyacrylamide gels to correspond to acetylcholinesterase 5 activity. Taking the $s^0_{20,w}$ of catalase as 11.4 and following the procedures of Martin and Ames [19], the $s^0_{20,w}$ of acetylcholinesterase 5 was computed to be 11.8 ± 0.2. The other peak corresponded to 7.4 S, but was shown by the gel-scanning technique on 10% gels, to represent the combined activities of acetylcholinesterases 1—4 with a smooth transition of activities dominated by the acetylcholinesterase 2 form. To reduce the error variances in the $s^0_{20,w}$ values of acetylcholinesterases 1—4 obtained under the above conditions, we repeated this experiment but with longer sedimentation times.

Fig. 5 shows the sedimentation behaviour of an aqueous DDT/S extract after 16-h centrifugation at 38 000 rev./min. With this centrifugation time the activity of acetylcholinesterase 1 was resolved, but the gel-scanning method [10] was re-

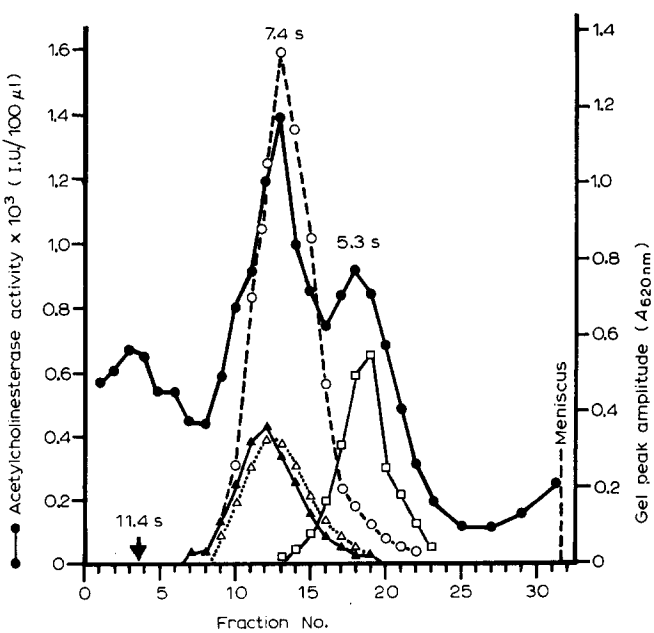

Fig. 5. Sucrose gradient centrifugation (38 000 rev./min for 16 h at 4°C) of an aqueous soluble DDT/S fly-head extract in a linear sucrose gradient (5—20% w/v) in 50 mM Tris · HCl (pH 7.5). Gradient preparation, fractionation, and acetylcholinesterase assays were performed as described in Materials and Methods. The solid line (●———●) shows the acetylcholinesterase activity in I.U./100 µl fraction. The peak near Fraction 3 corresponds to acetylcholinesterase 5 (11.8 S). Sedimentation of acetylcholinesterases 1—4, determined by the gel-scanning assay [10] on 10% polyacrylamide gels, is given as follows: acetylcholinesterase 1 (□———□); acetylcholinesterase 2 (○- - - - -○); acetylcholinesterase 3 (△. . . . .△); and acetylcholinesterase 4 (▲———▲). The arrow marks the peak sedimentation of the internal standard catalase (11.4-S).

quired to discriminate the distribution of acetylcholinesterase 2—4 activities.

The $s^0_{20,w}$ values were computed as follows: acetylcholinesterase 1, 5.3 ± 0.1; acetylcholinesterase 2, 7.4 ± 0.1; acetylcholinesterase 3, 7.8 ± 0.1; acetylcholinesterase 4, 8.1 ± 0.1. Thus, it seemed possible that Krysan and Chadwick [2] failed to separate the 5.3-S acetylcholinesterase 1 form because they used insufficient centrifugation time and because they depended on the Ellman technique [14].

To investigate the further possibility that preparation protocols may also have influenced the results, we examined the following extracts: (1) 1.5% w/v head concentration homogenized for 2.4 min at 20 000 rev./min in the phosphate buffer (pH 7.4) described by Krysan and Chadwick [2]; (2) 10% w/v head concentration homogenized for 2.5 min at 20 000 rev./min in the phosphate buffer (pH 6.8) of Krysan and Kruckeberg [1]. Following gradient centrifugation, polyacrylamide gel analysis revealed that both preparations contained acetylcholinesterase 1—5 forms with $s^0_{20,w}$ values as found earlier. Significantly, the 5.3-S acetylcholinesterase 1 form was characterized in both preparations.

For comparison with the molecular weights obtained by Krysan and Chadwick [2], we then used their assumptions to calculate molecular weight estimates for acetylcholinesterases 1—5, by substitution of our sedimentation coefficients into the Svedberg equation. The values obtained were: acetylcholinesterase 1, 117 000 ± 2000; acetylcholinesterase 2, 164 000 ± 3000; acetylcholinesterase 3, 172 000 ± 3000; acetylcholinesterase 4, 176 000 ± 3000; and acetylcholinesterase 5, 260 000 ± 5000. The results concur with our Ferguson plot data in that the acetylcholinesterase 1—5 family does not appear as a simple oligomeric series. More importantly, they provide convincing evidence that acetylcholinesterase 1, with a sedimentation coefficient of 5.3 S, indeed represents a smaller active component of acetylcholinesterase than any previously defined from fly heads.

(c) Sephadex G-150 chromatography. As a third method of molecular weight characterization, gel filtration on Sephadex G-150 was performed. Fig. 6 shows typical results obtained with an aqueous soluble extract. Three peaks of activity were resolved, although the first eluted peak of activity coincided with the void volume of the column. When examined by the gel-scanning method [10], these activity peaks were ascribed to acetylcholinesterase 5 (see later), acetylcholinesterase 2, and acetylcholinesterase 1 in order of elution. From the column calibration curve determined after Andrews [20], the molecular weights were estimated: acetylcholinesterase 5, $\geqslant$ 260 000; acetylcholinesterase 2, 160 000; and acetylcholinesterase 1, 80 000. Thus our observation that acetylcholinesterase 2 sedimented as 7.4 S and chromatographed as 160 000, agree with the findings of Krysan and Chadwick [2,3] and reinforce the conclusion that this component corresponds to the form postulated by Krysan and Kruckeberg [1] as the 'fundamental unit' of insect acetylcholinesterase. However, acetylcholinesterase 1 was again demonstrated as a smaller molecular component than acetylcholinesterase 2 and our interest now centered on the possibility that this form, rather than acetylcholinesterase 2, comprises the fundamental unit of fly-head acetylcholinesterase. The question remains why Krysan and Chadwick [2,3] failed to demonstrate acetylcholinesterase 1 using Sepha-

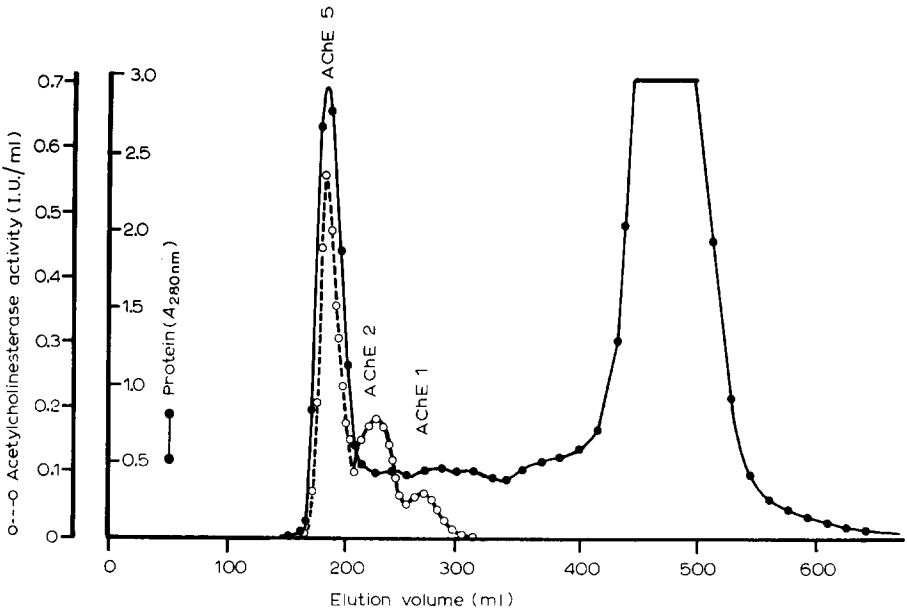

Fig. 6. Gel filtration of an aqueous soluble DDT/S fly-head extract (6 ml) on a Sephadex G-150 column (84 × 2.5 cm), equilibrated and developed at 4°C with 30 mM Tris · HCl (pH 8.0). Elution rate was 25 ml/h and fraction volume was 5 ml. The solid line is the absorbance at 280 nm; the dashed line refers to the acetylcholinesterase activity determined by the Ellman assay [14]; 92% of the applied activity was recovered. Contributing acetylcholinesterase forms were identified by a complementary gel-scanning assay [10]. AChE, acetylcholinesterase.

dex G-200 filtration. We believe they failed to resolve this component because they used a column of approximately half the length of our Sephadex G-150 column, and because they again depended solely on the Ellman assay [14].

Fig. 6 shows, in contrast to our sedimentation results (Fig. 5), that the major proportion of enzyme activity was obtained in higher aggregates than acetylcholinesterase 2. Electrophoretic analysis of this material excluded from Sephadex G-150, revealed acetylcholinesterase 5 as the dominant component. However, some activity was due to aggregate(s) which failed to penetrate 5% polyacrylamide gels; moreover, some activity was due to acetylcholinesterase 2. The gel-scanning technique on 10% gels showed that the latter could not be accounted by overlap of its fractionation range. Finally, electrophoresis failed to reveal any activity attributable to acetylcholinesterase 3 or to acetylcholinesterase 4. These anomalies may be partially explained on the assumption that, under our conditions, the lower forms tended to aggregate into acetylcholinesterase 5 and higher molecular form or forms. The anomaly of acetylcholinesterase 2 activity in the elution region of acetylcholinesterase 5 material may be explained differently on the basis of aggregate conversion as considered in the next section.

Convertibility of the multiple forms

(a) Autolysis. Krysan and Chadwick [3] showed that much of the particulate acetylcholinesterase of fly-head extracts could be solubilized by autolysis into a form chromatographically indistinguishable from the 'natively' soluble 7.3-S

form. If our 5.3-S acetylcholinesterase 1 form does indeed represent the fundamental unit, it might be expected that autolytic solubilization would yield this component as well as acetylcholinesterase 2.

A 20% w/v head preparation was autolysed at 25°C and pH 8.0 as described by Krysan and Kruckeberg [1]. Electrophoresis on 4—10% polyacrylamide gels revealed only one soluble component with acetylcholinesterase activity. Contrary to our expectations, this enzyme co-electrophoresed and chromatographed (Fig. 7) on Sepahdex G-150 as the acetylcholinesterase 2 form, in accordance with the findings of Krysan and Chadwick [3].

(b) Disulphide reduction. The results of autolysis indicate the conversion of higher aggregates to acetylcholinesterase 2. However, the failure to demonstrate acetylcholinesterase 1 remained unexplained. In an effort to resolve this anomaly, we treated both natively soluble extracts and the autolytic product with disulphide reducing agents. Our hypothesis was that formation of disulphide bonds may have promoted the conversion of acetylcholinesterase 1 into acetylcholinesterase 2; thus cleavage with disulphide reducing agents should reveal an increase in activity as acetylcholinesterase 1 with a corresponding decrease of acetylcholinesterase 2.

Fig. 8 shows the results obtained from reduction with 1 mM β-mercaptoethanol for 4 h at 30°C. In an attempt to prevent reassociation of disulphide bonds during electrophoresis [25], the reservoir buffers were made equimolar in β-mercaptoethanol with the treated samples. By these procedures, the activities of acetylcholinesterases 3—5 from an aqueous extract appeared unaltered, whereas the activity of acetylcholinesterase 2 was markedly reduced and that of acetylcholinesterase 1 was greatly increased relative to controls. With autolytic preparations, material from the single acetylcholinesterase 2 peak reacted

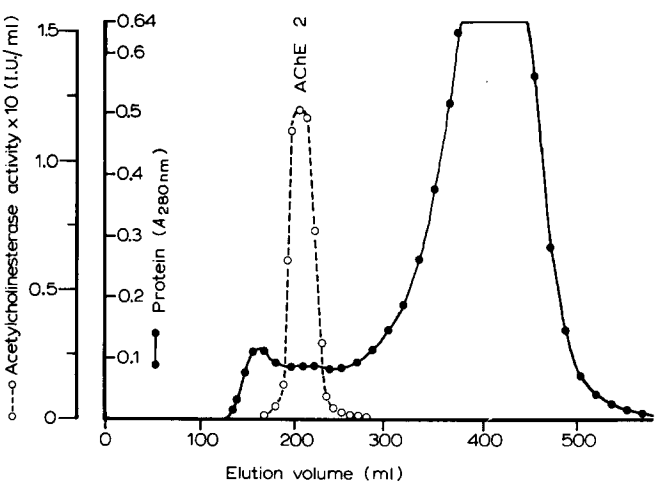

Fig. 7. Gel filtration on a Sephadex G-150 column (78 × 2.5 cm) of soluble acetylcholinesterase (5 ml) obtained by autolysis as described by Krysan and Kruckeberg [1]. The column was equilibrated and developed at 4°C with 30 mM Tris · HCl (pH 8.0) at 28 ml/h. The solid line refers to the absorbance at 280 nm; the dashed line refers to the acetylcholinesterase activity; 95% of applied activity was recovered. From the column calibration curve [20], the peak at 205 ml corresponded to a molecular weight of 160 000. AChE, acetylcholinesterase.

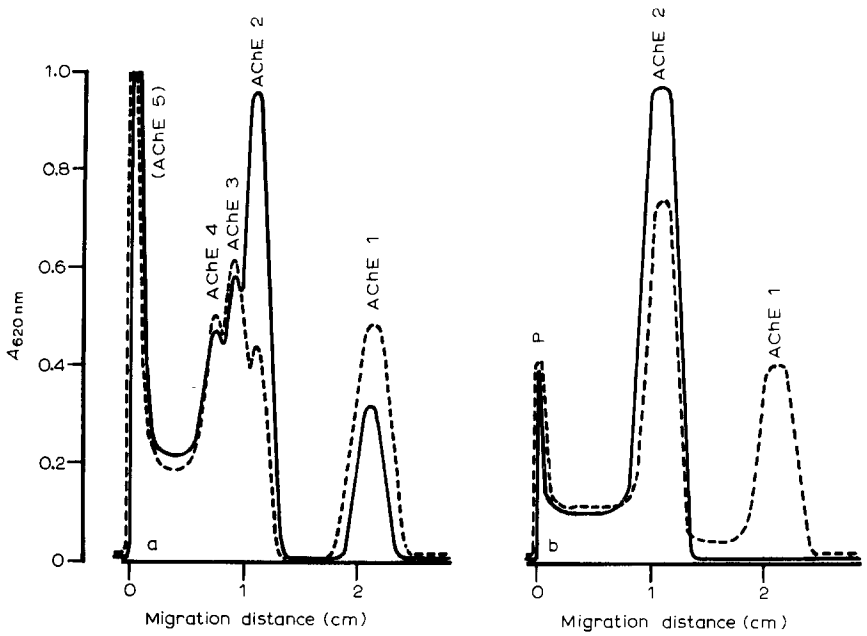

Fig. 8. Disulphide reduction of fly-head acetylcholinesterase with 1 mM β-mercaptoethanol. Reduced (- - - - -) and control (———) samples were electrophoresed on 10% polyacrylamide gels, stained in parallel, and then scanned at 620 nm: (a) reduction of an aqueous soluble extract; (b) reduction of autolysed enzyme from the Sephadex G-150 elution peak at 205 ml, Fig. 7. Each of the densitometric curves represents the mean of 4 replicates; P marks the optical dichotomy at the gel interface. AChE, acetylcholinesterase.

similarly. This conversion of acetylcholinesterase 2 into acetylcholinesterase 1 was subsequently found to be essentially instantaneous; immediate electrophoresis after addition of β-mercaptoethanol gave the same result.

To confirm the reduction of acetylcholinesterase 2 into acetylcholinesterase 1 and to avoid any possible sulphydryl reoxidation by excess ammonium persulphate in polyacrylamide gels, chromatographic studies of reduced preparations were made. An aqueous extract was reduced at pH 8.0 by 15 min exposure at 25°C to a large excess of dithiothreitol (10 mM). *N*-ethylmaleimide was then added directly to the medium in an amount which gave 5 mol/mol of dithiothreitol originally added. After 20 min reaction at 25°C, the alkylated sample was subjected to gel-filtration without prior dialysis.

Fig. 9 shows clearly the conversion of acetylcholinesterase 2 into acetylcholinesterase 1, and confirms by chromatography the results obtained by electrophoresis (Fig. 8). Whatever the reduction method, we were unable to effect the complete conversion of acetylcholinesterase 2 into acetylcholinesterase 1; experiments using higher concentrations (up to 50 mM) and longer reduction periods with the reducing agents β-mercaptoethanol, dithiothreitol, or thioglycollic acid still failed to achieve the complete reduction of acetylcholinesterase 2. Fig. 9 also shows a decrease in the activity of 'acetylcholinesterase 5'. This decrease, however, represents a minor proportion of activity and may represent experimental variation.

(c) Effect of tissue concentration. As stated earlier, zymograms of aqueous fly-head extracts were qualitatively identical. However, quantitative differences

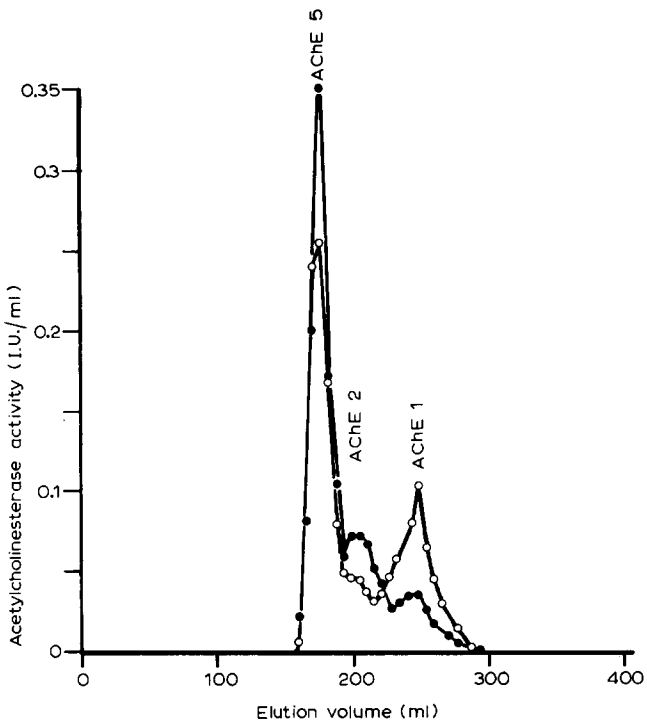

Fig. 9. Gel filtration of aqueous soluble fly-head acetylcholinesterase after disulphide reduction and alkylation as described in the text. Enzyme samples (4 ml) were chromatographed on a Sephadex G-150 column (78 X 2.5 cm) equilibrated and developed at 4°C with 30 mM Tris · HCl (pH 8.0) at 27.4 ml/h. Acetylcholinesterase (AChE) activities were determined by the Ellman assay [14]: control extract (●———●); reduced and alkylated extract (○———○). The gel-scanning assay [10] corroborated the elution patterns.

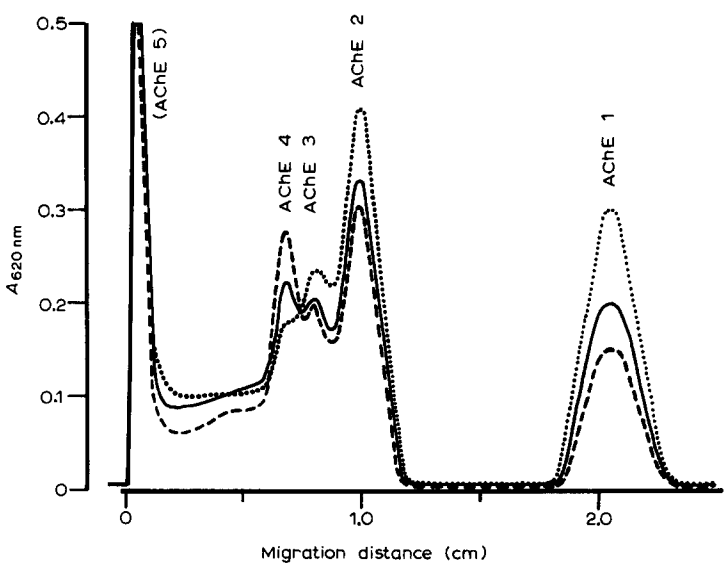

Fig. 10. Effect of tissue concentration on the relative proportions of acetylcholinesterases 1—5. Aqueous soluble extracts from 10% w/v (- - - - -), 20% w/v (———), and 30% w/v (. . . . .) homogenates were prepared from one batch of DDT/S fly heads; each extract was adjusted to 0.355 I.U./ml with glass-distilled water, then electrophoresed (50 µl/gel) and stained in parallel on 10% polyacrylamide gels. The densitometric curves represent the mean of 4 replicates; the variation about each curve was ⩽ ± 0.015 absorbance units at 620 nm.

in the relative activities of acetylcholinesterases 1—5 were observed between one preparation and another (for an example see Fig. 2). Tripathi et al. [11] have suggested such quantitative differences may be of physiological significance. It seemed more likely that these quantitative differences were the consequences of form conversion, and an investigation was made to determine whether tissue concentration at homogenization influenced our results. Fig. 10 shows that modifying the tissue concentration at homogenization does affect the relative activities of acetylcholinesterases 1—5. Aqueous homogenates of 30% w/v fly-head tissue released relatively greater activity in the lower molecular weight forms than was obtained at 10% w/v homogenization. This result supports the viewpoint of Krysan and Kruckeberg [1] that the relative activities of multiple forms bear no functional relationship to the physiological situation.

General discussion

Our results with the acetylcholinesterase of house-fly heads substantiate the evidence [2,3] that the enzyme can be isolated in various-sized aggregates. In aqueous extracts, the enzyme was shown by polyacrylamide gel electrophoresis to exist in five multiple forms. The higher aggregates in this series, and much of the particulate enzyme, were converted by the sequence of autolysis and disulphide reduction to the smallest active component, a 5.3-S form. This form of house-fly acetylcholinesterase has not been previously defined, and our results suggest that this form meets the criterion for the 'fundamental unit' of the enzyme.

The five forms observed here were characterized by molecular weight using Ferguson plot data and sedimentation coefficients, and by gel filtration calibration. The estimates obtained are presented in Table I.

The molecular weight estimate of approx. 160 000 for the 7.4-S acetylcholinesterase 2 form appears acceptably uniform by the three methods. The same holds true for the available estimates of acetylcholinesterase 3 and acetylcholinesterase 4. A wide discrepancy occurs between the estimates of acetylcholinesterase 5 molecular weight. We believe this discrepancy may be explained, at least in part, by asymmetry resulting from the attachment of 'tail' components

TABLE I

MOLECULAR WEIGHT ESTIMATES OF THE AQUEOUS SOLUBLE FORMS OF FLY-HEAD ACETYLCHOLINESTERASE

Form	Estimate by		
	Ferguson plot	Sedimentation	Gel filtration
Acetylcholinesterase 5	650 000	260 000	(⩾260 000)
Acetylcholinesterase 4	184 000	176 000	—
Acetylcholinesterase 3	174 000	172 000	—
Acetylcholinesterase 2	149 000	164 000	160 000
Acetylcholinesterase 1	80 000	117 000	84 000

[26—28]. This possibility will be examined further in a subsequent publication.

More important for our central concern are the estimates of the molecular weight of the 5.3-S acetylcholinesterase 1 form. The coincidence of estimates obtained by Ferguson plot and gel-filtration methods suggest the molecular weight of acetylcholinesterase 1 to be about 80 000. If this estimate is valid, then acetylcholinesterase 1 and acetylcholinesterase 2 with estimated molecular weights of 80 000 and 160 000, may represent respectively the monomer and dimer of a simple oligomeric series. The conversion of acetylcholinesterase 2 into acetylcholinesterase 1 by disulphide reducing agents (Figs. 8 and 9) conform with this supposition. This has led us to accept pro tem the molecular weight of 80 000 for acetylcholinesterase 1, rather than the 117 000 estimate obtained from its 5.3-S sedimentation coefficient.

Other estimates of the molecular weight of fly-head acetylcholinesterase appear to relate to our 7.4-S acetylcholinesterase 2 form. Thus sedimentation coefficients of 7.06, 7.3, and 7.56 S were reported by Yu [29], Krysan and Chadwick [2], and Campbell [29] respectively. Recently, Huang and Dauterman [30] obtained by gel filtration on Sepharose-6B, a molecular weight estimate of 209 000 for a highly purified fly-head preparation. To decide whether this acetylcholinesterase also conforms with the above four estimates, confirmation by sedimentation analysis would seem desirable.

Finally, the characterization of a 4.66-S form of acetylcholinesterase from heads of the house cricket [29], and a 5.46-S form from the American cockroach [29], corroborate the notion that a form of about 80 000 daltons constitutes the best candidate for the 'fundamental unit' of insect acetylcholinesterase.

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