# Structural Properties of Acetylcholinesterase from Eel Electric Tissue and Bovine Erythrocyte Membranes†

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ABSTRACT: Experiments are presented to determine some structural properties of eel and bovine erythrocyte acetylcholinesterase (AchE), purified by affinity chromatography. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of eel AchE demonstrated components weighing 60,000 and 75,000 g per mol. Both components were labeled with diisopropyl fluorophosphate (DFP) under conditions such that only active sites should be labeled. The 260,000-g/mol native molecule (Leuzinger, W., *et al.* (1969), *J. Mol. Biol. 40*, 217; Millar, D. B., and Grafius, M. A. (1970), *FEBS (Fed Eur. Biochem. Soc.) Lett. 12*, 61) therefore seems composed of two sets of two subunits, with each subunit bearing an active site. The properties of the erythrocyte esterase are as follows. The molecular weight of the native molecule is 200,000 g/mol by gel filtration. Sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated DFP-labeled subunits weighing 126,000 and 75,000 g per mol. These results suggest that the native molecule is a dimer composed of dissimilar subunits, both of which bear an active site. The amino acid composition of the red cell enzyme is unlike that of the eel enzyme, and the specific activity of the erythrocyte esterase is 2300 units/mg of protein as compared to at least 10,000 for the eel esterase. Thus, the structural properties of the two enzymes appear to differ considerably.

Acetylcholinesterase (AchE, EC 3.1.1.7), an almost completely membrane-bound enzyme of widespread tissue distribution (Koelle, 1963), was first completely purified from eel electric tissue (Leuzinger and Baker, 1967). AchE from both eel electric tissue and from bovine erythrocyte membranes was subsequently purified by affinity chromatography (Berman and Young, 1971), and other purification procedures for the eel enzyme, some employing affinity chromatography, have been presented (Millar and Grafius, 1970; Dudai and Silman, 1971; Rosenberry *et al.*, 1972). The molecular properties of the native eel enzyme have been well characterized (Leuzinger *et al.*, 1969). However, the subunit composition is still uncertain: the number of subunits has been estimated to be 4 (Kremzner and Wilson, 1968; Leuzinger *et al.*, 1969; Froede and Wilson, 1970) and 6 (Millar and Grafius, 1970), and the number of active sites per native enzyme molecule has been estimated to be 2 (Leuzinger, 1971), 3.3 (Rosenberry *et al.*, 1972), and 4 (Froede and Wilson, 1970; Mooser *et al.*, 1972). Furthermore, the molecular properties of the red cell enzyme are essentially unknown. Experiments using the eel and red cell enzymes purified by affinity chromatography (Berman and Young, 1971) are presented below to elucidate the subunit composition of the eel enzyme and some structural properties of the red cell enzyme.

## Materials and Methods

*Reagents.* All manipulations, unless otherwise noted, were carried out in "phosphate buffer" (0.1 M NaCl–0.01 M sodium phosphate, pH 7.5).

The following proteins were obtained from commercial sources: myoglobin (Pentex), bovine serum albumin (Sigma), human γ-globulin (fraction II, Sigma), catalase (Sigma), phosphorylase (Boehringer), partially purified eel acetylcholinesterase (type III, Sigma). Partially purified bovine erythrocyte AchE[1] was generously donated by Sigma Chemical Co.

Commercially prepared eel and erythrocyte AchE were further purified by affinity chromatography (Berman and Young, 1971). The previously described methods were modified as follows to ensure high yield and purity. Commercial enzyme preparations, 2 mg (eel) or 300 mg (red cell), were added to 1 ml (eel) or 10 ml (red cell) of cold phosphate buffer. The esterase suspensions were gently stirred for 15 min at 6°. The solutions were centrifuged at 100000*g* for 0.5 hr, and any precipitate was discarded. (In the case of the red cell enzyme, the supernatant solution was again centrifuged at 100,000*g* for 0.5 hr.) AchE in the supernatant solution from the final centrifugation was then purified by affinity chromatography. The esterase solution was added to agarose gels containing inhibitor I (eel) or inhibitor III (red cell) (Berman and Young, 1971) at a flow rate of 6–8 ml/hr. The resin–esterase complex was washed with cold phosphate buffer at 6–8 ml/hr until $A_{230}$ was less than 0.01. About 80% of the applied esterase activity is retained by the resin when these flow rates are used. To remove bound AchE, the resin–esterase complex was eluted at 3 ml/hr with ten column volumes of cold Tensilon solution (0.01 M Tensilon (Roche)–0.1 M NaCl–0.01 M sodium phosphate pH 7.5). About 60% of the resin-bound AchE is removed by this treatment if the following conditions are met. (1) The appropriate enzyme inhibitor is coupled to the extended side arm (Berman and Young, 1971) of 2% agarose immediately after both are prepared; (2) the agarose–inhibitor compound is itself used, for affinity chromatography, as soon as it is prepared. The yield of pure AchE may decrease if either condition is not satisfied. The purity of the esterase preparations was ascertained by polyacrylamide gel electrophoresis with the Tris–glycine

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[1] Abbreviations used are: AchE, acetylcholinesterase; Buch, butyrylcholine; DFP, diisopropyl fluorophosphate.

buffer system used previously (Berman and Young, 1971).

*Measurements of Enzyme Activity.* AchE was measured from the amount of 0.01 M NaOH needed to maintain constant pH during hydrolysis of acetylcholine at pH 7.5 and 22°. The incubation solvent contained 0.04 M $MgCl_2$–0.1 M NaCl–0.004 M acetylcholine chloride. One unit of enzyme activity represents 1 μmol of acetylcholine cleaved per min.

*Labeling with Diisopropyl Fluorophosphate (DFP).* Eel and bovine erythrocyte AchE was reacted with DFP in three ways. (1) Twenty units of pure enzyme was dissolved in 0.5 ml of cold phosphate buffer and 0.2 M butyrylcholine chloride (Buch, Eastman) (Cohen *et al.*, 1951), and this solution was shaken in the cold for 1 hr. This treatment is sufficient to prevent loss of enzymatic activity due to subsequent administration of DFP (see Results). The solution was made $10^{-6}$ M in [$^3$H]DFP (DFP*, Amersham-Searle). After being shaken for 2 hr, the solution was dialyzed to remove excess DFP*. The enzyme solution was then lyophilized in preparation for sodium dodecyl sulfate polyacrylamide gel electrophoresis (see below). After electrophoresis the gels were stained with Coomassie Brilliant Blue (1 hr), destained by diffusion (1 hr), and destained electrophoretically to visualize the subunit bands. The gels were then sliced, incubated with NCS (Zaitlin and Hariharasubramanian, 1970), and counted. (2) Twenty units of the pure enzyme in 0.5 ml of cold phosphate buffer was made $10^{-6}$ M in DFP*. The solution was shaken for 2 hr, excess DFP* was removed by dialysis, and the dialyzed solution was then treated exactly as in expt 1. (3) Twenty units of pure red cell AchE or commercially prepared eel AchE was dissolved in 0.5 ml of cold phosphate buffer and 0.2 M Buch, and was shaken in the cold for 1 hr. The solution was then made $10^{-6}$ M in unlabeled DFP, and was shaken in the cold for 2 hr further. Water (5 ml) was added to the reaction mixture to lower the ionic strength, and the resulting 5.5 ml was added to a 0.5-ml column of DEAE-cellulose (DE-52, Whatman) equilibrated with cold phosphate buffer. The protein was adsorbed to the resin, while the cationic blocking agent and excess DFP were unretarded. Protein was eluted from the resin with 5 ml of 0.4 M NaCl (erythrocyte enzyme) or 1.0 M NaCl (eel enzyme) at pH 7.5 *via* 0.01 M sodium phosphate buffer. The protein solution was dialyzed *vs.* cold phosphate buffer, made $10^{-6}$ M in DFP* and treated exactly as in expt 1.

*Electrophoresis of AchE Subunits.* Preparations of subunits of AchE were examined by electrophoresis on 5% polyacrylamide gels with the sodium dodecyl sulfate–phosphate buffer system described by Weber and Osborn (1969) except that 0.05 M sodium phosphate (pH 7.0) was used in both the gel and running buffers. Lyophilized protein preparations were dissolved in 30 μl of sodium dodecyl sulfate–mercaptoethanol–urea buffer (0.15 ml of $H_2O$; 0.10 ml of a solution of 0.5 M sodium phosphate, and 0.5% sodium dodecyl sulfate (pH 7.5); 0.25 g of urea; 0.05 ml of 2-mercaptoethanol), and the solutions were boiled for 3 min to inhibit protease activity (Pringle, 1970). Each boiled preparation was added to one gel. Bromophenol Blue served as the tracking dye and a constant current of 7.5 mA/gel was applied for 1.5 hr at 22°. Gels were stained for 3 hr with 0.25% Coomassie Brilliant Blue and destained electrophoretically (Weber and Osborn, 1969).

To establish that the plot of log ($M_w$) *vs.* migration distance is linear for the molecular weight range required, glyceraldehyde-3-phosphate dehydrogenase (37,000 g/mol; Klotz and Darnell, 1969) was cross-linked by reaction at pH 9.0 with a sixfold molar excess of glutaraldehyde, and the resulting polymers were electrophoresed with phosphorylase and catalase (57,500 g/mol; Klotz and Darnell, 1969). The plot was linear between 57,500 g/mol (catalase) and 220,000 g/mol (hexamer), with a correlation coefficient of 0.997.

The apparent molecular weights of the AchE subunits were estimated by electrophoresis with phosphorylase and catalase as markers.

*Amino Acid Composition and Partial Specific Volume.* About 50 μg of pure erythrocyte AchE was hydrolyzed with 6 N HCl in a sealed evacuated tube at 110°. Amino acid analyses were performed with a Beckman Model 120C amino acid analyzer by the methods of Crestfield *et al.* (1963). The partial specific volume for erythrocyte AchE was calculated from the amino acid composition by the method of Cohn and Edsall (1943) except that the specific volume of half-cystine was taken as 0.63 (McMeekin and Marshall, 1952). Because tryptophan was not determined for the red cell enzyme, tryptophan (mole %) was assumed to be the same as that for the eel enzyme (2%) (Leuzinger *et al.*, 1969).

*Gel Filtration.* The molecular weight of native red cell AchE was estimated by gel filtration with "phosphate buffer" on Sephadex G-200. For this purpose, myoglobin (17,200 g/mol; Weber and Osborn, 1969), bovine serum albumin (69,000 g/mol; Tanford *et al.*, 1967), human γ-globulin (160,000 g/mol; Dunker and Rueckert, 1969), catalase (232,000 g/mol; Klotz and Darnell, 1969), and eel AchE were used as markers. Because the elution profile of erythrocyte AchE was determined by activity measurements, impure red cell AchE could readily be used for these experiments.

*Protein Concentration of Erythrocyte AchE.* The Lowry technique (Bailey, 1967) was modified so that the small amounts of available pure red cell AchE could be measured accurately. The modification was simply to use 30% of the recommended quantities of reagents so that the protein concentration in the Lowry reaction mixture would be raised by 233%. Measured amounts of a bovine serum albumin solution were used as standards.

## Results

*Eel Acetylcholinesterase.* Eel AchE was purified by affinity chromatography (Berman and Young, 1971). AchE preparations that demonstrated one band upon Tris–glycine gel electrophoresis (Berman and Young, 1971) were further examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Two components are seen (Figure 1A). (Trace amounts of other polypeptide chains are occasionally also detectable; these may adhere to AchE in Tris–glycine buffer, but dissociate in sodium dodecyl sulfate buffer; 0.20 μg of marker protein can be clearly seen under these conditions.) The masses of the components were determined by electrophoresis in the presence of markers. The larger component appears approximately midway between the phosphorylase and catalase markers (92,500 and 57,500 g per mol; Klotz and Darnell, 1969), while the smaller component migrates just behind catalase. The average molecular weights of the components are estimated to be 75,000 g/mol (range for three experiments: 76,500–73,000) and 60,000 g/mol (range 60,500–59,500).

To establish which of the two components shown in Figure 1A contains the enzymatically active site(s) of AchE, the protein was treated with DFP*. DFP has been shown by competitive inhibition studies to bind to the active site(s) of AchE (Augustinsson and Nachmansohn, 1949; Cohen *et al.*, 1951). To correct for nonspecific binding of DFP, butyrylcholine, an acetylcholine analog that should protect active sites from DFP (Cohen *et al.*, 1951), was used in conjunction

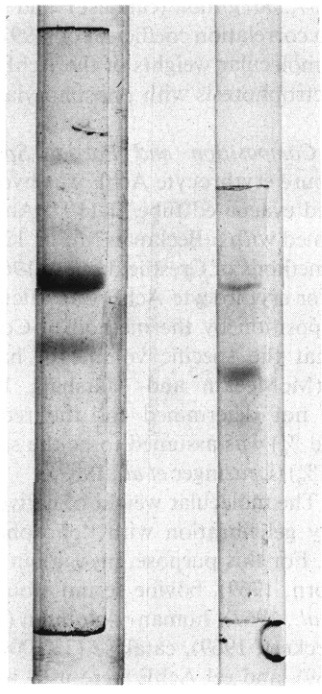

FIGURE 1: Sodium dodecyl sulfate gel electrophoretic patterns of AchE. Direction of migration is from top to bottom. (A, left) Pure eel AchE (4 $\mu$g) was added to the gel; (B, right) pure erythrocyte AchE (4 $\mu$g) was added to the gel.

with the phosphorylating agent. Preliminary experiments revealed that protection did occur for both the bovine and eel enzymes. Butyrylcholine, and then DFP, were added to an erythrocyte AchE solution as described above (expt 3). After DEAE chromatography the recovery of enzyme activity was 20–30%. The resin's capacity for irreversible protein absorption may be comparable to the small amount (<10 $\mu$g) of esterase used, and may be the reason for the low per cent recovery. Recovery from solutions to which neither ligand had been added was similarly 20–30%. Buch therefore completely protected the enzyme from inhibition by DFP. However, when pure eel esterase was used, the recoveries in the two preliminary experiments were only about 5%. For this reason, commercially available impure eel AchE was used for labeling (expt 3). The recovery of activity from (impure) eel enzyme solutions to which Buch and DFP had been added, or to which neither ligand had been added, was 60–70%. Thus, the eel enzyme is also protected by Buch from the phosphorylating agent. (The presence of impurities should not affect the DFP* labeling pattern for the eel enzyme, since DFP* binding sites on the impurities should react with the previously administered unlabeled DFP.)

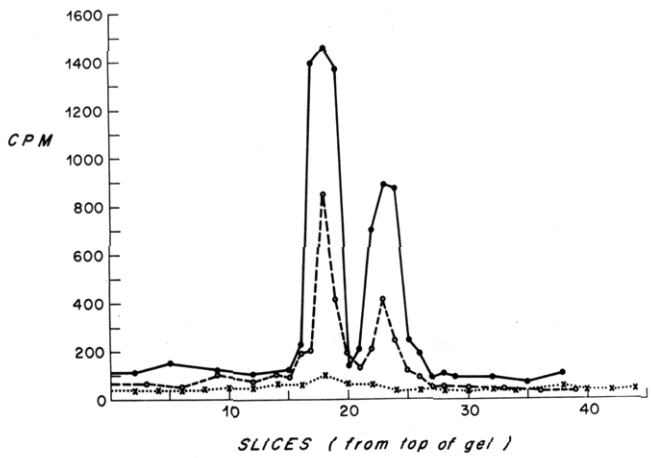


FIGURE 2: Labeling of eel AchE subunits with radioactive DFP. Eel AchE was labeled with radioactive DFP (DFP*) in three ways (see Materials and Methods). *Dotted line* (expt 1): AchE was reacted with DFP* in the presence of butyrylcholine. *Solid line* (expt 2): AchE was reacted with DFP* in the absence of butyrylcholine. *Dashed line* (expt 3): AchE was reacted with DFP in the presence of butyrylcholine; the excess of both ligands was removed by DEAE chromatography; the enzyme was then reacted with DFP*. One slice = 1.5 mm.

The results of the three experiments in which eel AchE was treated with DFP are shown in Figure 2. Incubation of the enzyme with DFP* in the presence of butyrylcholine resulted in no labeling of either component (dotted line, Figure 2). Incubation of the enzyme with DFP* in the absence of Buch resulted in labeling of both components (solid line, Figure 2). Comparison of the two labeling patterns indicates that the groups protected by Buch from DFP* are present on both components. This conclusion is supported by the third experiment recorded in which only sites that are protected are labeled (dashed line, Figure 2).[2]

These results are similar to those of Dudai and coworkers (Dudai and Silman, 1971; Dudai *et al.*, 1972a), who found that pure AchE from trypsin-treated electric tissue could be dissociated into DFP-labeled subunits weighing 88,000 and 64,000 g per mol. The Sigma type III enzyme used here was prepared by Lawler's (1959) method (first ammonium sulfate fraction), which does not involve deliberate proteolysis, and the difference in molecular weight determinations could be related to the trypsin treatment by Dudai *et al.* However, proteolysis may also occur during preparation of the Sigma enzyme.[3] In both experiments the heavier band appears to contain more protein and more DFP*. The reason for this discrepancy is not clear.

*Bovine Erythrocyte Acetylcholinesterase: Molecular Weight of Native Esterase.* Since insufficient amounts of pure erythrocyte AchE were available for equilibrium sedimentation studies, the molecular weight of native red cell AchE was estimated by gel filtration on Sephadex G-200. Myoglobin, albumin, and $\gamma$-globulin emerge from G-200 columns such that the elution volume is virtually proportional to log (molecular weight) (Andrews, 1965), and a reliable calibration plot should be determined by the values of $V/V_0$ *vs.* log ($M_w$) for these proteins. It was hoped that proteins of higher molecular weights would similarly be useful markers. However, eel AchE emerged in a broad band more quickly and catalase emerged more slowly than would be expected. The discrepancy for eel AchE could well be due to aggregation of the

[2] The decreased amounts of total labeling in the third experiment with respect to the second experiment are explained by the 30–40% loss of enzyme that occurs when Buch is removed by DEAE chromatography.

[3] Grafius and coworkers (Grafius and Millar, 1965, 1967; Grafius *et al.*, 1968), Massoulié *et al.* (1970), and Dudai *et al.* (1972a,b) have shown that the soluble AchE in pressed or homogenized electric tissue exists in >60S, 18S, 14S, 11S, and 8S forms. The non-11S forms are generally convertible to 11S forms (which may not be identical: Grafius *et al.*, 1971) by prolonged cold storage, trypsin treatment, or lipase treatment (Grafius and Millar, 1965; Grafius *et al.*, 1971; Massoulié *et al.*, 1970; Dudai *et al.*, 1972a). It is possible that the 11S form studied here results from reaction of electroplax protease or lipase with the various esterase species in the electroplax homogenate.

TABLE I: Amino Acid Composition of Eel and Erythrocyte AchE.

| Amino Acid | Mol %: Eel Enzyme[a] | Mol %: Erythrocyte Enzyme[b] |
|---|---|---|
| Asp | 11.6 | 8.0 |
| Thr | 4.5 | 5.4 |
| Ser | 6.9 | 10.9 |
| Glu | 9.2 | 11.2 |
| Pro | 8.1 | 4.7 |
| $^1/_2$-Cys | 1.1 | 0.8[c] |
| Gly | 8.1 | 13.2 |
| Ala | 5.8 | 10.6 |
| Val | 6.9 | 7.3 |
| Met | 2.9 | 1.5 |
| Ile | 3.4 | 2.8 |
| Leu | 9.2 | 7.5 |
| Tyr | 4.0 | 3.2 |
| Phe | 5.8 | 3.5 |
| Lys | 4.5 | 6.2 |
| His | 2.2 | 1.6 |
| Arg | 5.8 | 4.4 |

[a] Calculated from the data of Leuzinger *et al.* (1969). Tryptophan was omitted for this calculation. [b] Tryptophan was not determined. [c] This value is somewhat low because some protein cystine and cysteine is degraded during acid hydrolysis.

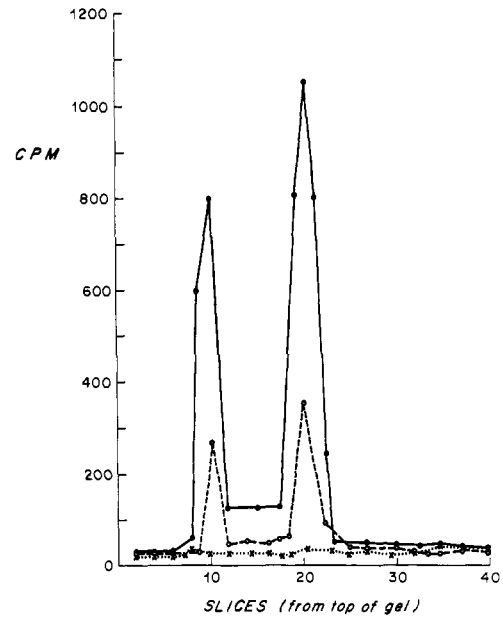


FIGURE 3: Labeling of erythrocyte AchE subunits with radioactive DFP. Pure erythrocyte AchE was labeled with radioactive DFP (DFP*) in three ways (see Materials and Methods). *Dotted line* (expt 1): AchE was reacted with DFP* in the presence of butylcholine. *Solid line* (expt 2): AchE was reacted with DFP* in the absence of butyrylcholine. *Dashed line* (expt 3): AchE was reacted with DFP in the presence of butyrylcholine; the excess of both ligands was removed by DEAE chromatography; the enzyme was then reacted with DFP*. One slice = 1.3 mm.

esterase in the approximately 0.1 M salt solution used, since aggregation in similar solutions has been repeatedly reported (for example, Grafius and Millar, 1965; Dudai *et al.*, 1972b). Therefore, the calibration line that was used was based on the data for the three proteins of lower molecular weights. The elution profile of erythrocyte AchE showed a sharp, symmetrical peak with a $V/V_0$ value of 1.49, from which an apparent molecular weight of 199,000 g/mol was calculated. The shape of the red cell AchE peak indicates that this enzyme, unlike the eel enzyme, does not aggregate in 0.1 M NaCl solution.

*Subunit Composition of Red Cell Acetylcholinesterase.* Figure 1B shows that when 4 $\mu$g of pure erythrocyte AchE is electrophoresed with 5% polyacrylamide gels containing sodium dodecyl sulfate buffer, two bands are detected (0.2 $\mu$g of marker protein can be clearly seen under these conditions). When the protein was electrophoresed in the presence of the molecular weight markers phosphorylase (92,500 g/mol) and catalase (57,500 g/mol), the esterase bands show molecular weights of 126,000 g/mol (range for three experiments 122,000–128,000) and 75,000 g/mol (range 74,000–76,000).

To determine which subunits contain active sites, erythrocyte AchE was treated with DFP. Incubation of the red cell esterase with DFP* in the presence of butyrylcholine resulted in no labeling of either subunit (dotted line, Figure 3). Reaction of the enzyme with DFP* in the absence of Buch led to about equal labeling of the subunits (solid line, Figure 3). Comparison of the two labeling patterns indicates that the groups protected by Buch are about equally distributed between the subunits. This conclusion is supported by the third experiment recorded in which only protected sites are labeled (dashed line, Figure 3). Both subunits appear to contain sites protected by Buch.[4]

*Specific Activity and Turnover Number of Erythrocyte AchE.* The modified Lowry technique (see Materials and Methods) was used to elucidate the specific activity of red cell AchE. Twelve units of activity corresponded to 5.1 $\mu$g of protein, yielding a specific activity of 2300 units/mg of protein. The turnover number of erythrocyte AchE can be calculated from the specific activity (2300 $\mu$mol of acetylcholine cleaved per min per mg of protein), the molecular weight (200,000 g/mol), the subunit composition of the enzyme (1 mol of enzyme/2 mol of subunits), and the assumed number of active sites per subunit (1). The turnover number is $2.3 \times 10^5$ mol of acetylcholine cleaved per mol of active site per min. This value is close to the values previously estimated from the number of acetylcholine molecules cleaved per molecules of DFP bound to impure bovine red cell enzyme preparations: $2.8 \times 10^5$ (Cohen *et al.*, 1955), $2.9 \times 10^5$ (Cohen and Warringa, 1953), and $3.7 \times 10^5$ (Cohen *et al.*, 1953).

*Amino Acid Composition and Partial Specific Volume of Red Cell AchE.* The results of the amino acid analysis are listed in Table I. The comparison between the mole per cent of each amino acid in eel and in erythrocyte AchE indicates that the amino acid compositions of the two proteins differ considerably.

The partial specific volume of bovine erythrocyte AchE, assuming mole % tryptophan = 2%, is 0.725, with SD = 0.003.

[4] The decreased amount of total labeling in the third experiment with respect to that in the second experiment is explained by the 70–80% loss of enzyme that occurs when the blocking agent is removed by DEAE chromatography.

## Discussion

*Eel AchE.* Leuzinger *et al.* (1969) have previously shown that the eel enzyme used for these studies[3] has a native molecular weight of 260,000 g/mol. Millar and Grafius (1970) report a molecular weight of 259,200 g/mol for a similar preparation. The purpose of the present work was to clarify the subunit composition of this enzyme. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the pure enzyme shows subunits weighing 75,000 and 60,000 g per mol. No evidence is apparent for a single subunit weighing 42,200 g/mol, as suggested by Millar and Grafius (1970), or for a single species of about 50,000 g/mol as suggested by Froede and Wilson (1970). Both types of subunits are labeled with DFP* under conditions where binding occurs only to sites protected by butyrylcholine. If we assume that Buch only protects esteratic groups, then the DFP*-labeling pattern indicates that both types of subunits contain an active site. The simplest hypothesis is that the 260,000 g/mol of native esterase consists of four subunits, two of mol wt 75,000 g/mol and two of mol wt 60,000 g/mol, and that each subunit bears an active site.

The hypothesis of four subunits is in accord with work of Leuzinger *et al.* (1969) in which two C-terminal glycine residues and two C-terminal serine residues per native molecule were found, and of that of Froede and Wilson (1970), who found the subunit molecular weight of radioactively labeled AchE to be one-quarter that of the labeled native enzyme. The hypothesis of four active sites is in disagreement with one report indicating only two active sites per 260,000-g/mol species (Leuzinger, 1971). The initial rate of reaction of AchE with an acetylcholine analog demonstrated 2 mol of ligand cleaved per mol of native enzyme. On the other hand, most reports indicate approximately four active sites per native molecule. Froede and Wilson (1970) found about 4 mol of DFP bound to 1 mol of AchE, and Rosenberry and Bernhard (1971) and Mooser *et al.* (1972) obtained evidence for four active sites by titration of AchE with a fluorescent probe. Dudai and Silman (1971) observed DFP-labeled subunits of mass 88,000 and 64,000 g per mol. These experiments, similar to those reported herein, have been discussed in the Results section. An intermediate value, 3.3 active sites/native enzyme molecule, has been proposed by Rosenberry *et al.* (1972). These workers determined $E_{280}^{1\%}$ for eel AchEt by five methods, and found that the determinations ranged from 17.6 to 21.8. The value of $E_{280}^{1\%}$ from dry weight and refractive index measurements (18.0) was judged the most reliable and was used to compute the Figure 3.3. Yet, the extinction coefficient might be more accurately estimated by analysis of nitrogen content. In this case, the extinction coefficient would be 21.6 (Rosenberry *et al.*, 1972), and the number of active sites would be 4.0.

*Erythrocyte AchE.* The native molecular weight of bovine erythrocyte AchE is close to 200,000 g/mol, judged by gel filtration studies. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the pure enzyme indicates that the esterase is a dimer, with subunits weighting 126,000 and 75,000 g per mol. These results differ from prior conclusions from sodium dodecyl sulfate gel electrophoresis of DFP-labeled human erythrocyte membranes (Bellhorn *et al.*, 1970). When the membranes were incubated with sodium dodecyl sulfate in the absence of mercaptoethanol, the label migrated with a molecular weight of 180,000 g/mol; this was taken to be the weight of the native enzyme. When mercaptoethanol was added to the incubation mixture, the label migrated with a molecular weight of 90,000 g/mol; this was taken to be the weight of each of the two subunits. The difference in native molecular weights between the prior work and the present work may be explained by the calibration problems inherent in the method used previously. The absence of mercaptoethanol and the presence of large amounts of membrane protein may have caused the enzyme to migrate not in strict proportion to its molecular weight. The disparity in subunit molecular weight determination is difficult to explain.

If we assume that butyrylcholine only protects esteratic groups in the red cell esterase, the DFP–labeling experiments indicate that each subunit contains an active site. It is unusual that an enzyme has subunits with apparently the same function, binding DFP and Buch, but with widely different molecular weights. It is possible that proteolytic cleavage during preparation of the commercially available enzyme had artificially lowered the molecular weight of the smaller subunit.

The amino acid composition of the red cell enzyme is dissimilar to that of eel enzyme. The turnover number for the erythrocyte esterase is $2.3 \times 10^5$, whereas the value for the eel enzyme is near to $6.5 \times 10^5$ (Rosenberry *et al.*, 1972).[5]

Thus, there appears to be considerable structural dissimilarity between the two acetylcholinesterases, even though apart from the difference in turnover numbers there is general similarity in their kinetic properties (Augustinsson and Nachmansohn, 1949; Nachmansohn and Rothenberg, 1945; Iverson and Mann, 1969).

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[5] This calculation is based on the 260,000-g/mol native esterase containing four active sites and having a specific activity of 10,100 units/mg of protein (Rosenberry *et al.*, 1972).

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# The Variable Subunit Structure of Lysine-Sensitive Aspartylkinase from *Escherichia coli* TIR-8†

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ABSTRACT: Lysine-sensitive aspartylkinase (AK III) has been purified to homogeneity by the criteria of disc gel electrophoresis at pH 8.3, electrophoresis in sodium dodecyl sulfate, and by sedimentation velocity. It has been shown to be composed of similar subunits of 48,000 ± 4000 molecular weight by sedimentation equilibrium of reduced, carboxymethylated enzyme in 6 M guanidine hydrochloride and by gel electrophoresis in 0.1% sodium dodecyl sulfate. Equilibrium sedimentation, analytical G-200 Sephadex chromatography, and $s_{20,w}$ plus $D_{20,w}$ determinations have shown that AK III may be obtained in two forms differing in quaternary structure. One form is a fairly stable dimer of about 100,000 molecular weight, $s_{20,w}$ = 6.6 S. The second form appears to be in a dimer–tetramer equilibrium and at higher protein concentration behaves as a tetramer of about 200,000 molecular weight, $s_{20,w}$ = 10.1 S. The partial specific volume of the protein determined by equilibrium sedimentation in $D_2O$ is 0.746 cm³/g. The $E^{1\%}_{280}$ was determined by dry weight to be 3.60. The specific activity of the enzyme increases during log-phase growth and reaches a sharp maximum at the onset of stationary phase. The enzyme purified from late stationary phase exhibits a high uv absorbancy below 280 nm. Lysine addition to native enzyme causes a difference spectrum with a peak at 294 nm. Titration of the native enzyme at 294 nm exhibits an apparent cooperative transition with the half-maximal change occurring at 0.2 mM lysine.

Lysine-sensitive aspartylkinase is one of three aspartylkinases found in *Escherichia coli* $K_{12}$. Most of our knowledge of these enzymes comes from the work of G. N. Cohen and his collaborators, and this work has recently been definitively reviewed (Cohen, 1969). Two of these enzymes are complex proteins which also carry homoserine dehydrogenase activity. Aspartylkinase I (AK I–HSDH I),[1] which is inhibited by threonine, was isolated by Truffa-Bachi *et al.* (1968) and reported to consist of six similar subunits of 60,000 molecular weight. Recently, Starns *et al.* (1972), studying *E. coli* $K_{12}$ λ, have reported a subunit molecular weight of about 85,000, indicating that AK I–HSDH I is composed of four similar subunits. Aspartylkinase II (AK II–HSDH II), the methionine repressed

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[1] Abbreviations used are: AK III, lysine-sensitive aspartylkinase; Gdn·HCl, guanidine hydrochloride; HSDH, homoserine dehydrogenase.