Characterization of Lipid-Protein Interactions in Acetylcholinesterase Lipoprotein Extracted from Bovine Erythrocytes

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Acetylcholinesterase was released from bovine erythrocytes in hypo-osmotic sodium phosphate buffer. Initially, about 30% of the enzyme was released in a soluble lipoprotein form, and further incubation resulted in the progressive release of the enzyme in a particulate form. Solubilization of the acetylcholinesterase in the particulate fraction with Lubrol WX (2mg/ml) resulted in the loss of all lipids except a non-exchangeable fraction identified as cardiolipin. Addition of a mixture of erythrocyte phospholipids to the soluble forms and to the Lubrol WX-solubilized enzyme resulted in the formation of particulate forms of the enzyme with increased partial specific volume and Stokes radius, and a break in the Arrhenius plot of the enzyme activity around 20°C. The break in the Arrhenius plot was abolished by treatment of a soluble enzyme preparation with 1.8M salt (NaCl) in phosphate buffer, conditions that allowed the extraction of cardiolipin from the enzyme by chloroform/methanol. Failure of the high-salt treatment to decrease the Stokes radius made it unlikely that the bound cardiolipin formed a boundary layer or annulus around the protein. It is suggested that cardiolipin is bound to the core of the dimeric protein structure, thereby controlling the acetylcholinesterase activity.

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is an integral protein of the erythrocyte membrane (Mitchell & Hanahan, 1966; Aloni & Livne, 1974; Jackson & Whittaker, 1972; Heller & Hanahan, 1972). The enzyme is extracted from human erythrocyte-membrane 'ghosts' by detergents (Jackson & Whittaker, 1972; Heller & Hanahan, 1972) or high-salt treatment (Mitchell & Hanahan, 1966; Heller & Hanahan, 1972), but high-ionic-strength treatment fails to release the enzyme from intact erythrocytes (Aloni & Livne, 1974). Bovine erythrocyte acetylcholinesterase appears to be less firmly bound to the membrane, as it is also released in hypo-osmotic media (Burger *et al.*, 1968). Acetylcholinesterase released from both human and bovine erythrocytes behaves as a lipoprotein (Mitchell & Hanahan, 1966; Heller & Hanahan, 1972; Sihotang, 1976; Niday *et al.*, 1977), with a partial specific volume higher than expected from its amino acid composition (Berman, 1973; Beauregard & Roufogalis, 1977). Acetylcholinesterase was selectively extracted from intact human erythrocytes in the presence of phospholipid vesicles (Bouma *et al.*, 1977) and from pig and ox erythrocytes by a phosphatidylinositol-specific phospholipase C (Low & Finean, 1977). The enzyme has been shown to re-associate with lipid membranes (Jain *et al.*, 1973) and to be incorporated into lipid monolayers (Wiedmer *et al.*, 1978).

The minimum molecular weight of bovine erythrocyte acetylcholinesterase was 72000 by X-ray-inactivation analysis at 10°C (Levinson & Ellory, 1974). Partially purified bovine erythrocyte acetylcholinesterase was dissociated by sodium dodecyl sulphate into two subunits of mol.wts. 126000 and 75000, both of which contained active sites (Berman, 1973). Bellhorn *et al.* (1970) and Niday *et al.* (1977) found mol.wt. values of 180000 and 160000 respectively for human erythrocyte acetylcholinesterase, which further dissociated into two equal subunits after electrophoresis in sodium dodecyl sulphate. Multiple forms of human erythrocyte acetylcholinesterase were obtained after Triton X-100 solubilization (Wright & Plummer, 1973).

In a previous study (Beauregard & Roufogalis, 1977) we suggested that cardiolipin tightly associated with bovine erythrocyte acetylcholinesterase was associated with a break in the Arrhenius plot, by modulation of the rate-limiting substrate-induced conformational change in the enzyme active site (G. Beauregard & B. D. Roufogalis, unpublished work). The interaction of cardiolipin with the enzyme was functionally disrupted by treatment with 1.8M-NaCl (Beauregard & Roufogalis, 1977), but only in Ca²⁺-chelating conditions (G. Beauregard & B. D. Roufogalis, unpublished work). In the present study we report on the nature of the acetylcholinesterase

species extracted from bovine erythrocytes in hypo-osmotic media, the influence of lipids on the enzyme activity and the changes in the physicochemical properties of the enzyme before and after high-salt treatment in phosphate buffer.

Experimental

Enzyme extraction

Bovine blood was obtained at a local slaughterhouse and preserved in 0.38% sodium citrate; 1 litre of cells was packed by centrifugation at 4500g for 10 min. The supernatant and buffy coat were removed by aspiration. The packed cells were washed twice in iso-osmotic 0.155M-NaCl and the final volume of packed cells was recorded (around 250 ml). Then 1 vol. (50 ml) of packed cells was haemolysed twice in 7 vol. of 8.93 mM (25 mosM)-sodium phosphate buffer, pH 7.4. The 'ghosts' were harvested at 20000g and the supernatant fractions kept for analysis of acetylcholinesterase activity. The 'ghosts' were incubated in the same volume of 25 mosM-phosphate buffer, pH 7.4, for periods up to 48 h at 5°C. At various intervals (see the Results section) the membranes were separated by centrifugation at 20000g and the acetylcholinesterase activity in the supernatant was determined. Increasing amounts of acetylcholinesterase were solubilized during the process.

Assay of acetylcholinesterase

Acetylcholinesterase was assayed by a radiometric assay, as previously described (Roufogalis *et al.*, 1973). The assay medium contained 1 mM-acetylcholine iodide, including 19.9 nCi of [^{14}C]acetylcholine iodide, in 0.1 M-sodium phosphate, pH 7.4. Relative activities are reported as net c.p.m. after correction for non-enzymic-hydrolysis blanks. Enzyme concentrations were chosen such that reaction rates were linear with time and enzyme concentration. Arrhenius plots were determined as described previously (Beauregard & Roufogalis, 1977).

Solubilization with Lubrol WX

A particulate fraction of acetylcholinesterase was extracted from 'ghosts' by hypo-osmotic lysis and pelleted by centrifugation in a Beckman SW 41Ti rotor for 3 h at 5°C and 100000g_{av.}. The particulate fraction was partially purified by solubilization with Lubrol WX (2 mg/ml) in 2 mM-phosphate buffer (pH 7.4) at 5°C for 12–16 h, as previously described (Beauregard & Roufogalis, 1977).

Extraction of lipids, and reconstitution

Lipids were extracted from either packed erythrocytes or the particulate fraction of acetylcholinesterase

with chloroform/methanol (1:1, v/v), as described by Reed *et al.* (1960). Lipids extracted from 1 ml of packed erythrocytes or from the particulate fraction from 40 ml of packed erythrocytes were resuspended in 11.5 ml of 2 mM-phosphate buffer, pH 7.4. The suspension was mixed with 0.5 ml of Lubrol WX-solubilized enzyme or soluble fraction from the hypo-osmotic extraction and treated in a Sorvall Omni-Mixer for 2 min at 5°C and at setting 8. The mixture was incubated for 1 h at room temperature (20°C) and then for 12–16 h at 4°C, and the resulting particulate fraction was pelleted by centrifugation for 3 h at 5°C and 100000g_{av.}.

Phospholipids were identified by chromatography on precoated silica-gel 60F-254 sheets (20 cm × 20 cm; EM Laboratories, Elmsford, NY, U.S.A.) with chloroform/methanol/30% (w/v) NH₃ (14:6:1, by vol.). Phospholipid standards were run on the same sheet, and detected with Rhodamine 6G (Supelco, Bellefonte, PA, U.S.A.). Bovine mitochondrial cardiolipin, bovine phosphatidylcholine and phosphatidylethanolamine, and plant phosphatidylinositol were from Applied Science Laboratories (State College, PA, U.S.A.) and bovine phosphatidylserine was from Supelco.

Gel filtration

A column (2.6 cm × 100 cm) of Sepharose 6B (Pharmacia, Uppsala, Sweden) was equilibrated and eluted with 0.2 M-NaCl/2 mM-sodium phosphate, pH 7.4, at 4°C, unless otherwise stated. The flow rate was maintained at about 7 ml/h with a Desaga peristaltic pump. Columns were calibrated with 50 mg of catalase and 10 mg of bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.), 10 mg of aldolase, 20 mg of ribonuclease and 1 mg of Blue Dextran (Pharmacia). Enzyme and standards were introduced into the flow tube through a three-way tap. Fractions (3 ml) were collected for analysis. Bovine serum albumin, aldolase and ribonuclease were determined by the A_{280} , and Blue Dextran by the A_{650} . Catalase was measured by a modification of the method of Beers & Sizer (1952), by the decrease in A_{240} of 0.5% H₂O₂ in 0.1 M-sodium phosphate, pH 7.4. Sucrose was used to determine the final volume of the column (V_t) (487 ml) and Blue Dextran to determine the void volume (V_0) (156 ml). The elution volume (V_e) of the acetylcholinesterase and the standards was used to determine K_d (Andrews, 1970) as follows:

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

Plots of (K_d)⁺ against known Stokes radii (Andrews, 1970) were used to obtain the Stokes radius of the acetylcholinesterase preparation.

Sedimentation coefficient

Sedimentation coefficients were determined in linear 5–20% (w/v) sucrose gradients in 2mM-sodium phosphate, pH 7.4. Marker enzymes and acetylcholinesterase were placed at the top of the gradient and centrifuged in a Beckman SW 41Ti rotor in a Beckman L2-65B ultracentrifuge at 4°C for the time and speed indicated in the Figure legend. Fractions (nine drops) were collected by carefully lowering a capillary into the tubes at the end of the run and pumping the liquid from the bottom with a Desaga peristaltic pump. When necessary, the refractive indices were also determined to correct the apparent sedimentation coefficient for the density difference between acetylcholinesterase and the marker proteins (Martin & Ames, 1961). Aldolase and bovine serum albumin standards were determined at 280 nm.

Equilibrium sedimentation

The method was developed to measure the partial specific volume ($\bar{v}$) of the lipoproteins. Acetylcholinesterase in a partially preformed sucrose gradient was centrifuged for approx. 66 h at 4°C and 147000g in a Beckman SW 41Ti rotor. The partially preformed sucrose gradient was made by layering 1.3 ml (measured by weight) of each of the following sucrose concentrations (M) in 2mM-sodium phosphate, pH 7.4; 2.696, 1.604, 0.978, 0.624, 0.408, 0.276, 0.195, 0.140, 0.105, 0.083. Refractive indices were determined with a Fisher refractometer at 22°C.

Materials

Lubrol WX was from Sigma Chemical Co.; DEAE-Sephadex was from Pharmacia. Inorganic salts and solvents were of analytical-reagent grade. Partially purified bovine erythrocyte acetylcholinesterase, obtained from Sigma, was a freeze-dried powder of 20 mg of acetylcholinesterase, 100 mg of gelatin and 30.7 mg of NaCl in 5 ml of sodium phosphate buffer, pH 7.6. [^{14}C]Acetylcholine iodide (1–5 Ci/mol) was from New England Nuclear (Lachine, Que., Canada). Cation-exchange resin (Na^+ form, CGC 241, 200–400 mesh) was from J. T. Baker (Phillipsburg, NJ, U.S.A.).

Results

Extraction of acetylcholinesterase from bovine erythrocytes

Various conditions for the release of acetylcholinesterase from bovine erythrocytes were examined. Incubation of bovine erythrocytes in a hypo-osmotic medium (25 mosM-sodium phosphate, pH 7.4) resulted in the progressive release of acetylcholinesterase activity from the haemolysed cells (Table 1, *a*).

Table 1. Release of soluble and particulate forms of acetylcholinesterase from bovine erythrocytes

Washed erythrocytes were lysed as described in the Experimental section. At the various times shown the 20000g supernatant was centrifuged at 100000g_{av} for 3 h. The acetylcholinesterase activity in the supernatant and pellet (resuspended to the original volume) was determined by the radiometric assay. The results shown are typical experiments from five blood samples.

Extraction time (h)	Acetylcholinesterase activity (% of total)		
	Cumulative release*	Soluble	Particulate
(a) 25 mosM-Sodium phosphate, pH 7.4			
0.25	32	84	16
0.25	39	61	39
47.5	135	1	99
(b) 25 mosM-CaCl ₂			
0.25	10	77	23
0.25	12	46	54
15.5†	14	25	75
5.0	16	7	93
17.0	19	22	78
95.0	22	0	100
+7.7 mM-EDTA			
97.5	30	6	94
116.0	36	7	93
(c) 1.2 M-NaCl			
19.0	7	70‡	30
92.5	28	96‡	4

* Calculated as the percentage of activity measured in intact erythrocytes.

† After initial lysis in 25 mosM-CaCl₂, the membranes were suspended in 25 mosM-sodium phosphate, pH 7.4, for further incubation.

‡ The centrifugation time was increased to 6 h at 100000g_{av} to compensate for the increase in density of 1.2 M-NaCl.

Release of acetylcholinesterase was considerably decreased in the presence of CaCl₂, approaching a maximum release of only about 25% of the total enzyme activity in 95 h (Table 1, *b*). The inhibition of release was overcome by the addition of EDTA. Enzyme was also released by incubation of erythrocytes in high salt (1.2 M-NaCl) (Table 1, *c*).

The acetylcholinesterase released from the membranes could be separated into 'soluble' and 'particulate' species, according to its behaviour on centrifugation at 100000g_{av} for 3 h. The proportion of soluble and particulate fractions at various times after hypo-osmotic incubation are given in Table 1. Whereas the acetylcholinesterase released in the first two washes in 25 mosM-sodium phosphate (approx. 30%) was predominantly a soluble form, the remaining enzyme released after prolonged incubation

(47.5 h) in phosphate buffer was almost completely a particulate form. A similar pattern was obtained in the presence of CaCl_2 (Table 1, *b*). By contrast, the acetylcholinesterase released by high-salt treatment (1.2M-NaCl), even after 92.5 h, was almost entirely in the soluble form (Table 1, *c*). Both the soluble and particulate enzyme forms showed a similar low activity toward butyrylcholine iodide to that of purified Sigma acetylcholinesterase, in a radiometric assay.

Characterization of acetylcholinesterase released from bovine erythrocytes

Acetylcholinesterase released from erythrocytes (25 mosm-sodium phosphate, pH 7.4, for 20 h) was resolved into two separate peaks of enzyme activity by equilibrium sedimentation in a preformed sucrose gradient (Fig. 1). The two species exhibited $\bar{v}$ values of 0.90 and ≤ 0.77 ml/g. By contrast, the particulate fraction obtained after centrifugation of the extracted enzyme at $100000g_{av}$ for 3 h gave a single peak of enzyme activity with a $\bar{v}$ of 0.90 ml/g (Beauregard & Roufogalis, 1977). The proportion of enzyme activities in the two peaks obtained in Fig. 1 was 28% high-density form and 72% low-density form, consistent with the ratio of soluble and particulate forms of the enzyme found in this particular preparation (results not shown). Equilibrium sedimentation of soluble forms of acetylcholinesterase in $^2\text{H}_2\text{O}$ showed heterogeneous species that were variable in different preparations, and were not examined further. However, the $\bar{v}$ found for all species was consistently higher than 0.725 ml/g, the value expected from the amino acid composition of the enzyme in the absence of

Fig. 1. Equilibrium sedimentation of a 20000g supernatant fraction of acetylcholinesterase released from erythrocytes after 20h

The sucrose gradient was partially preformed in 2mM-sodium phosphate, pH 7.4, and centrifuged as described in the Experimental section. A plot of $\log[\text{sucrose}]$ versus (distance)² confirmed the linear relationship expected (Mahler & Cordes, 1973). Each fraction consisted of nine drops (0.39 ml).

lipid (Berman, 1973). Heterogeneity was also found on Sepharose 6B chromatography and high-speed sedimentation on a sucrose gradient (results not shown).

The sedimentation behaviour of the particulate form of acetylcholinesterase is shown in Fig. 2. The sedimentation profile shows a broad distribution of activity indicative of heterogeneity. At the two peaks of activity the $s_{20,w}^0$ values were 21.2S and 23.7S, after correction for the difference in $\bar{v}$ of acetylcholinesterase and the marker enzymes (Martin & Ames, 1961). Consistent with the large sedimentation coefficients, the particulate form was eluted in the void volume of a Sepharose 6B column. The physicochemical properties of the acetylcholinesterase released from bovine erythrocytes after hypo-osmotic lysis are summarized in Table 2.

Solubilization of particulate acetylcholinesterase released from bovine erythrocytes

Particulate fractions of acetylcholinesterase released from bovine erythrocytes after hypo-osmotic lysis were solubilized with Lubrol WX. The effect of Lubrol WX concentration is shown in Fig. 3. Maximum solubilization of the enzyme activity (after centrifugation at $100000g_{av}$ for 3 h) occurred at around 2mg of Lubrol WX/ml, and the recovery of enzyme activity remained constant up to 10mg/ml. Above this concentration the acetylcholinesterase activity was decreased. In our hands, 0.2mg of Triton X-100 or sodium deoxycholate/ml destroyed all enzyme activity of the particulate fraction. Extraction of the particulate fraction with Lubrol WX (2mg/ml) decreased $\bar{v}$ from 0.90 to 0.79 ml/g (Table 2). The properties of Lubrol WX-solubilized acetylcholinesterase partially purified by DEAE-Sephadex

Fig. 2. Sedimentation of the particulate fraction of acetylcholinesterase

The sedimentation coefficient was determined relative to β -galactosidase (β -gal) and catalase (cat) after sedimentation for 17.4h (see the Experimental section). Each fraction consisted of nine drops (0.39 ml).

as described in the Experimental section are summarized in Table 2. The corrected $s_{20,w}^0$ (7.3S) and Stokes radius (6.0nm) are similar to those of the soluble forms extracted from erythrocytes (results not

shown) and a commercial preparation obtained from Sigma (Table 2).

Reconstitution of soluble forms with phospholipids

Lipids extracted from a particulate fraction of acetylcholinesterase were added to the Lubrol WX-solubilized enzyme. Although the reconstitution procedure resulted in a low yield of enzyme activity (34% of the starting activity), most of this activity (66.4%) was in the particulate form after centrifugation at $100000g_{av}$ for 3 h. $\bar{v}$ for the reconstituted particulate form increased from 0.79 to 0.92ml/g (Table 2). The physicochemical properties of the reconstituted enzyme are summarized in Table 2. The reconstituted enzyme was heterogeneous on sucrose-gradient sedimentation, with major peaks of $s_{20,w}^0$ 10.4S and 20.5S (results not shown). A particulate form of acetylcholinesterase also resulted when lipids extracted from a particulate fraction were added to soluble forms extracted from bovine erythrocytes (results not shown).

Phospholipid analysis

The phospholipid composition of the particulate fraction of acetylcholinesterase extracted from bovine

Fig. 3. Solubilization of acetylcholinesterase from the particulate fraction by Lubrol WX

The particulate fraction was incubated with Lubrol WX for approx. 16h at 4°C. The percentage of soluble activity was determined by comparison of the acetylcholinesterase activity in the $300000g_{av}$ supernatant with the total activity in the particulate and supernatant fractions.

Table 2. Physicochemical properties of acetylcholinesterase

The methods used are described in the Experimental and Results sections. Molecular weights and frictional coefficient ratio (f/f_0) were calculated as described in Mahler & Cordes (1973). A mol.wt. of 144000 for acetylcholinesterase dimer was obtained from Levinson & Ellory (1974). A cardiolipin mol.wt. of 1388 and density of 1.00g/ml (Rand & Sengupta, 1972) was used in the calculation of the mol of cardiolipin associated with the enzyme, from the differences in the volume of various enzyme forms. A $\bar{v}$ of 0.725ml/g calculated from the amino acid composition of erythrocyte acetylcholinesterase (Berman, 1973) was used in the calculations. Cardiolipin was the only phospholipid considered in the calculation.

Enzyme form	$\bar{v}$ (ml/g)	Stokes radius (nm)	$s_{20,w}^0$ (S)	f/f_0	Mol.wt.	Phospholipid content (mol/mol of enzyme)
Particulate	0.90	Void volume	21.2, 23.7*			
Lubrol WX-solubilized	0.79	6.0	7.3	1.42	242000	†
Lubrol WX-solubilized enzyme plus total lipid extract	0.92	Void volume	10.4, 20.5*			
Sigma	0.81	4.7	7.7	1.13	215000	50
Sigma in 1.8M-NaCl	0.78	4.8	7.1	1.27	169500	19
and 2mM-sodium phosphate	-0.83‡				-212000	-50§

* Two peaks of enzyme activity were found in the sucrose gradient (see Fig. 2).

† Assuming 50mol (69338g) of cardiolipin was bound, as in the Sigma enzyme (Beauregard & Roufogalis, 1977), 52500g of Lubrol WX is bound per mol of enzyme dimer, by using a density of 1.09 (Uesugi *et al.*, 1971).

‡ Values of 0.82–0.83ml/g were obtained from three experiments in which the enzyme was layered in fraction 9 (from the top) of the preformed sucrose gradient in 1.8M-NaCl plus phosphate, and values of 0.78ml/g and ≤ 0.79 ml/g were obtained when the dialysed enzyme was layered on a 69% sucrose gradient (centrifuged for 66h at $100000g$) or a preformed sucrose gradient in 2mM-sodium phosphate respectively.

§ Direct phosphate analysis of the cardiolipin spot on a thin-layer chromatogram showed that 33mol was extracted by chloroform/methanol in high salt and phosphate.

erythrocytes was similar to the phospholipid composition of the whole membrane, whereas only cardiolipin is found in the Lubrol WX-solubilized enzyme (Beauregard & Roufogalis, 1977). Extraction of 290 units of Sigma acetylcholinesterase/6ml with chloroform/methanol yielded three phospholipid spots, corresponding to phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine (or phosphatidic acid) (Table 3). Re-extraction of the remaining protein with chloroform/methanol in the presence of 1M-NH₃ or extraction of a concentrated sample of the enzyme (290 units/ml) resulted in the further removal of cardiolipin (Table 3). No choline-containing lipids were detected, in contrast with their presence in extracts of erythrocyte membrane lipids.

Effect of phospholipids on catalytic activity of acetylcholinesterase

The particulate form of acetylcholinesterase exhibits a non-linear Arrhenius plot, with a break at around 20°C (Beauregard & Roufogalis, 1977), whereas a linear Arrhenius plot was obtained for the soluble forms extracted from the erythrocyte (Fig. 4). The Lubrol WX-solubilized enzyme from the particulate fraction and the soluble enzyme from Sigma retains the non-linear Arrhenius plot (Beauregard & Roufogalis, 1977). This break was abolished after treatment of these enzymes with 1.8M-NaCl in 2mM-sodium phosphate, pH 7.4 (Beauregard & Roufogalis, 1977). Addition of lipids extracted from bovine erythrocytes to the soluble enzyme forms released from the bovine erythrocytes resulted in a particulate

form that had regained the break in the Arrhenius plot at around 20°C (Fig. 4).

Fig. 4. Arrhenius plots of acetylcholinesterase released from bovine erythrocytes

The supernatant from a 300000g_{av}. centrifugation of the initial fraction of acetylcholinesterase released from bovine erythrocytes in hypo-osmotic medium was separated into soluble fraction 1 ($\bar{v}$ = 0.80 ml/g) (●) and soluble fraction 2 ($\bar{v}$ ≥ 0.88 ml/g) (□) by equilibrium sedimentation. Lipids extracted from the particulate form of acetylcholinesterase (from 40 ml of erythrocytes) were added to a mixture of soluble fractions 1 and 2, as follows (○). A portion of the soluble fractions was mixed with 11.5 ml of 2mM-sodium phosphate, pH 7.4, and the extracted lipids. The suspension was stirred at speed 8 in a Sorvall Omni-Mixer for 2 min in ice. After 1 h at 20°C the mixture was allowed to stand for 16 h at 4°C and then centrifuged at 100000g for 3 h at 5°C. Bars represent the range of duplicate determinations.

Table 3. Phospholipid composition of various fractions of bovine erythrocyte acetylcholinesterase

Phospholipids were extracted as described in the Experimental section. Phospholipid standards were applied separately on the same thin-layer chromatogram for each of the experiments shown. Spots were determined with Rhodamine 6G.

Phospholipid	<i>R_F</i>							
	Standard	Erythrocyte membrane	Sigma (290 units/ml)	Standard	Sigma (290 units/6ml)	Sigma (290 units/6ml) + NH ₃ *	Standard	Sigma (290 units/ml)
Phosphatidylinositol	0.11	—	0.11	—	0.13	—	0.08	0.10
Phosphatidylserine	—	0.19	0.20	—	0.19	—	0.19	0.19
Sphingomyelin	—	0.27	—	—	—	—	—	—
Phosphatidylcholine	0.35	0.37	—	0.36	—	—	0.35	—
Phosphatidylethanolamine	0.40	0.46	0.43	0.47†	0.5	—	0.39	0.41
Phosphatidic acid	—	—	—	0.47†	—	—	—	—
Cardiolipin	0.54	—	0.53	0.59	—	0.58	0.54	0.53

* The enzyme solution was made 1M in NH₃ after the initial chloroform/methanol extraction, and re-extracted with chloroform/methanol.

† Phosphatidylethanolamine and phosphatidic acid were not resolved on these chromatograms.

Effect of high-salt treatment in phosphate buffer on the properties of soluble acetylcholinesterase

The changes in the physical properties of acetylcholinesterase occurring as a result of treatment with high salt in sodium phosphate was examined on the partially purified soluble enzyme from Sigma. This enzyme exhibited physical properties and behaviour similar to those of the enzyme extracted from bovine erythrocytes. The elution behaviour on Sepharose 6B for Sigma acetylcholinesterase (A), Lubrol WX-solubilized acetylcholinesterase (B) and Sigma acetylcholinesterase treated with 1.8 M-NaCl in 2 mM-sodium phosphate buffer, pH 7.4 (C), is shown in Fig. 5. The Stokes radius of the Lubrol WX-solubilized enzyme (6.0 nm) was significantly greater than that of the soluble species (Table 2). Treatment of the Sigma enzyme with high salt in phosphate did not appreciably alter the Stokes radius (from 4.7 nm to 4.8 nm) (Table 2). The determination of sedimentation coefficients of these species by comparison to marker proteins is shown in Fig. 6. High-salt treatment in sodium phosphate resulted in a decrease of the $s_{20,w}^0$ of the Sigma enzyme from 7.7 to 7.1 S (Table

Fig. 5. Gel filtration through Sepharose 6B

Values of Stokes radii of β -galactosidase (β -Gal; 6.9 nm), apoferritin (APF; 6.1 nm), catalase (Cat; 5.2 nm), aldolase (ALD; 4.59 nm), bovine serum albumin dimer (BSA_2) (4.35 nm) and monomer (BSA; 3.49 nm) and ribonuclease A (RNAase A; 1.79 nm) were taken from Sober (1970). The calibration curve ($\square$) was obtained in 0.2 M-NaCl/2 mM-sodium phosphate, pH 7.4. Sigma acetylcholinesterase was eluted as a single peak at the position shown by arrow A. The Lubrol WX-solubilized enzyme was eluted at arrow B in the column equilibrated in the same solution with Lubrol WX (2 mg/ml). The Sigma enzyme treated with 1.8 M-NaCl in 2 mM-sodium phosphate, pH 7.4, was eluted at arrow C in a column equilibrated with the same solution and calibrated with ribonuclease A, bovine serum albumin monomer and dimer (Δ).

Fig. 6. Speed sedimentation profiles of Sigma acetylcholinesterase (a), Lubrol WX-solubilized acetylcholinesterase (b) and Sigma acetylcholinesterase treated with 1.8 M-NaCl and 2 mM-sodium phosphate, pH 7.4 (c)

Sedimentation coefficients were determined in 5–20% linear sucrose gradients. Fraction size was nine drops (0.39 ml). (a) Sigma acetylcholinesterase (0.2 mg) in 0.05 M-NaCl/2 mM-sodium phosphate, pH 7.4; 100 000g at 20°C for 17 h. (b) Lubrol WX-treated acetylcholinesterase (0.2 mg) in 2 mM-sodium phosphate, pH 7.4, and Lubrol WX (2 mg/ml); 100 000g at 4°C for 16.5 h. (c) Sigma acetylcholinesterase (0.2 mg) treated with 1.8 M-NaCl and 2 mM-sodium phosphate, pH 7.4; 200 000g at 4°C for 18 h. $\bullet$, Refractive index of sucrose gradient; Δ , sedimentation coefficients of standards (Cat, catalase; ADH, alcohol dehydrogenase; Ald, aldolase; BSA, bovine serum albumin). $\circ$, Acetylcholinesterase activity.

2). The partial specific volume was essentially unaltered from 0.81 (Table 2). From the changes in physical properties of the enzyme with high-salt treatment in phosphate, the frictional coefficients, molecular weights and cardiolipin contents were calculated and are summarized in Table 2.

Discussion

Acetylcholinesterase is firmly associated with the erythrocyte membrane (Aloni & Livne, 1974) and catalyses the hydrolysis of acetylcholine on the outer

surface of the membrane (Herz *et al.*, 1963; Firkin *et al.*, 1963). The enzyme activity can be released from erythrocyte 'ghosts', but not from intact erythrocytes, by alterations in the ionic strength. In the present study we have confirmed previous reports that acetylcholinesterase species released from erythrocyte membranes behave as lipoproteins (Burger *et al.*, 1968; Mitchell & Hanahan, 1966; Niday *et al.*, 1977).

Acetylcholinesterase released during the hypo-osmotic lysis of bovine erythrocytes was separated into soluble and particulate forms, according to their sedimentation on centrifugation at 300 000 $g_{av.}$. The soluble form was preferentially released in the early phase of the hypo-osmotic lysis, whereas the particulate form was released after prolonged hypo-osmotic lysis in 25 mosM-sodium phosphate buffer, pH 7.4. Both forms of the enzyme activity were lipoproteins, as determined by their partial specific volumes, and both forms exhibited heterogeneity on Sepharose 6B column chromatography and sucrose-gradient centrifugation. Soluble forms of acetylcholinesterase were also extracted from bovine splanchnic nerve and adrenal medulla (Chubb & Smith, 1975) as a similar percentage of total enzyme activity found in the present study (20–30%; five determinations). The soluble forms from splanchnic nerve and adrenal medulla were shown to be isoenzymic forms of a single detergent-solubilized species and did not appear to be derived as artifacts. It is not known whether the soluble forms obtained from bovine erythrocytes represent enzyme less firmly associated with the membrane than the bulk enzyme, or a structurally distinct isoenzyme. Despite their lipoprotein nature, the soluble forms did not give the non-linear Arrhenius plot characteristic of the presence of certain lipids on the membrane-bound enzyme (Beauregard & Roufogalis, 1977; Aloni & Livne, 1974; Kumamoto *et al.*, 1971). However, addition of lipids extracted from the erythrocyte membrane to the soluble forms resulted in a particulate form exhibiting the characteristic Arrhenius-plot break at around 20°C. This supports the suggestion that the soluble fraction represents an enzymic form of acetylcholinesterase less firmly associated with the membrane structure, rather than a distinct molecular species. The soluble forms were true acetylcholinesterase, as determined from the very low extent of hydrolysis of butyrylcholine. The relative proportions of soluble and particulate forms did not appreciably differ in old and new erythrocytes, separated by the method of Murphy (1973) (results not shown).

Acetylcholinesterase activity in the particulate fraction was solubilized by extraction in Lubrol WX. Solubilization occurred with the concomitant loss of lipids, as demonstrated by the decrease in the partial specific volume and by direct phospholipid analysis. From the Stokes radius and the sedimentation co-

efficient of the Lubrol WX-solubilized acetylcholinesterase, we calculated that the mol.wt. increased by 52 500 as a result of the binding of Lubrol WX to the dimer of mol.wt. 144 000 (Levinson & Ellory, 1974). The association of Lubrol WX with the enzyme increased its Stokes radius from 4.7 to 6.0 nm, and the frictional-coefficient ratio from 1.13 to 1.42, suggesting formation of a coat of detergent around the enzyme. Removal of phospholipid from acetylcholinesterase by sodium deoxycholate was also reported by Sihotang (1976). The Lubrol WX-solubilized acetylcholinesterase, like the soluble enzyme obtained commercially from Sigma, exhibited a break in the Arrhenius plot of acetylcholine hydrolysis. Both these enzyme preparations contained cardiolipin. The Sigma enzyme also contained phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine, although the presence of these lipids appears to vary in different enzyme batches. The latter lipids appeared to be loosely associated by predominantly hydrophobic interactions, since they were readily extracted by chloroform/methanol and did not appear in the Lubrol WX preparation solubilized from the membrane. [It is noteworthy that these phospholipids are associated with the inner half of the erythrocyte membrane bilayer (Zwaal *et al.*, 1975), despite the extracellular orientation of the enzyme catalytic site (Herz *et al.*, 1963; Firkin *et al.*, 1963). The diameter of 9.3 nm determined from the Stokes radius (including water of hydration) is compatible with penetration of the enzyme to the inner half of the bilayer.] By contrast, cardiolipin appeared to be tightly bound to acetylcholinesterase by predominantly ionic interactions, since it could be extracted from the Sigma or Lubrol WX-solubilized enzyme by chloroform/methanol only in the presence of 1 M-NH₃ or 1.8 M-NaCl in sodium phosphate, pH 7.4 (Beauregard & Roufogalis, 1977) or from Sigma enzyme concentrated to 290 units/ml (which has a high concentration of salts and phosphate owing to their presence in the commercial Sigma preparation).

In addition to facilitating cardiolipin removal by chloroform/methanol, high-ionic-strength treatment of acetylcholinesterase abolished the break in the Arrhenius plot of acetylcholine hydrolysis. Although there are many possible reasons for non-linear Arrhenius plots in enzymes (Dixon & Webb, 1958), the results suggested that the loss of the break was due to the disruption of the cardiolipin-acetylcholinesterase complex. The changes in physico-chemical properties of Sigma acetylcholinesterase after high-salt treatment in phosphate buffer were examined. High-salt treatment did not disrupt the dimer or cause aggregation of the dimer. It was calculated from the hydrodynamic properties that 19–50 molecules of cardiolipin were associated with the enzyme, assuming a $\bar{v}$ of 0.725 ml/g calculated from the amino acid composition (Berman, 1973).

The presence of tightly bound phospholipid in a ratio of $0.3 \pm 0.1 \mu\text{g}/\mu\text{g}$ of enzyme, has also been reported by Sihotang (1976) for human erythrocyte acetylcholinesterase, similar to the ratio of cardiolipin by weight found in the present study (0.33:1). Tightly bound cardiolipin that could be removed by chloroform/methanol extraction in 1 M-NH₃ (Awasthi *et al.*, 1971) has been found associated with Triton X-100-solubilized cytochrome *c* oxidase (Vik & Capaldi, 1977). It is of interest to consider the location of the tightly bound lipid on acetylcholinesterase. We tentatively conclude from its inaccessibility to chloroform/methanol extraction that the cardiolipin modulates acetylcholinesterase activity when bound to the core of the dimeric structure.

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