The Purification of Cholinesterase from Horse Serum

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A relatively simple method is described by which cholinesterase was purified about 19000-fold starting from horse serum. Typically 20 litres of serum were processed to yield 15–18 mg of electrophoretically pure cholinesterase in the form of an active salt-free dry powder. The method included two stages: fractionation with $(\text{NH}_4)_2\text{SO}_4$ and ion-exchange chromatography. The $(\text{NH}_4)_2\text{SO}_4$ stage included, in principle, the acid (pH 3) step of the Strelitz (1944) procedure. The step took advantage of the stabilizing effect that 33% satd. $(\text{NH}_4)_2\text{SO}_4$ has on cholinesterase activity at pH 3 and it is recognized that in the absence of $(\text{NH}_4)_2\text{SO}_4$ the enzyme is rapidly destroyed at pH 3. Cholinesterase was significantly more stable to pH 3.0 at 2°C than at 24°C, and the acid step was done at both temperatures. The specific activities of the final products obtained by way of acid steps were the same at either temperature, thus indicating that the step has not harmed the enzyme active sites. The product from the first two stages was purified over 18000-fold and was 85–90% cholinesterase. The remaining impurities were removed by preparative gel electrophoresis. The product was about 40% more active and contained 40% more active sites per unit weight than electrophoretically pure cholinesterase prepared from partially purified commercial starting material. Although the number of active sites per molecule was not determined with certainty, a value of at least 3 and possibly 4 was indicated. The partial specific volumes were determined with a precision density meter, on the ultracentrifuge and from the amino acid and carbohydrate composition. The values by these independent methods were 0.688, 0.71 and 0.712 ml/g, respectively. The amino acid and carbohydrate composition was determined. The cholinesterase contained 17.4% carbohydrate including 3.2% *N*-acetylneuraminic acid.

Although significant progress has been made in developing procedures for purifying horse serum cholinesterase (acetylcholine acyl-hydrolase; EC 3.1.1.8) (e.g. Jansz & Cohen, 1962; Heilbronn, 1962; Lee & Harpst, 1971; Main *et al.*, 1972), electrophoretically pure enzyme in the quantities needed for definitive characterization studies is still not available. The procedures have followed the first stages of the Strelitz (1944) method, either directly or by the use of partially purified commercial material prepared by this procedure. The use of the Strelitz (1944) method has raised the possibility, although by no means the certainty, of another problem concerned with the quality of the cholinesterase in the purified preparations. The key step in the Strelitz (1944) method uses 2.5M- H_2SO_4 to lower the serum supernatant to pH 3, 33% saturated with respect to $(\text{NH}_4)_2\text{SO}_4$. The cholinesterase remains active and in solution. However, it does not appear to be widely appreciated that horse cholinesterase in serum is not stable at pH 3 in the absence of high concentrations of $(\text{NH}_4)_2\text{SO}_4$. A review of the first stages

of the Strelitz (1944) method with this in mind clearly raises the possibility of partial inactivation of cholinesterase. Partial inactivation would, in turn, lead to low estimates of the number of active sites per molecule. Since the number of active sites is vital to a correct understanding of both the kinetic and structural properties of the enzyme, the question of quality is as crucial as that of quantity. One is reminded of the words of Cohen & Oosterbaan (1963) made in connexion with cholinesterase: 'It should be remembered, however, that the enzymes, crystallized or otherwise purified, are often the sorry results of a series of maltreatments to which a protein has been subjected on its way from its natural biological position...into the test tube.'

In the present paper a procedure is described which started from horse serum, rather than from the partially purified commercial preparations used previously (Main *et al.*, 1972). A preliminary study established the conditions under which cholinesterase was stabilized at pH 3. The procedure was based on an appreciation of these conditions and was sufficiently flexible to permit variations to be made by which the effect of the pH 3 step on the quality of the highly purified product could be further evaluated.

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During the course of the investigation, it became evident that the time and effort involved in carrying the procedure through to completion would be critical to its success. A small number of relatively simple, reproducible steps seemed essential. With this in mind, the procedure was limited to the use of two separation techniques: fractionation with $(\text{NH}_4)_2\text{SO}_4$ and ion-exchange chromatography on DEAE-Sephadex. The efficiency required to effect a purification in the order of 20000-fold with a small number of steps was achieved in part by selective elution of DEAE-Sephadex columns with linear choline chloride gradients (Yoshida, 1970). The product was about 90% pure. Electrophoretically pure cholinesterase was obtained by preparative polyacrylamide-gel electrophoresis (Main *et al.*, 1972). Sufficient purified cholinesterase (15–20mg) was obtained to permit valid estimates of purity. The efficiency of the method together with the quality of the product (a water-soluble salt-free active freeze-dried powder) suggested that the method could lead to the production of the quantities needed for definitive structural studies.

Experimental and Results

Materials

Horse serum (frozen, non-sterile, type 2, imported) was from Pel Freez Biologicals, Rogers, Ark., U.S.A. Choline chloride and butyrylthiocholine were from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. DEAE-Sephadex A-50 was from Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A. Acrylamide, bisacrylamide (*NN'*-methylenebisacrylamide), TEMED (*NNN'*-tetramethylethylenediamine) and ammonium persulphate were electrophoresis grade from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Other chemicals and reagents were obtained from the usual commercial sources.

Measurement of protein concentration

Protein was determined as a routine by $E_{280}^{1\text{cm}}$ measurements. Protein was also determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a reference and by drying to constant weight over P_2O_5 after freeze-drying.

Measurement of cholinesterase activities

The method of Ellman *et al.* (1961) was used as a routine except that butyrylthiocholine was substituted for acetylthiocholine. One unit of activity was the hydrolysis of $1\mu\text{mol}$ of 1mM -butyrylthiocholine/min at 25°C in 0.1M -sodium phosphate buffer at pH 8.1. When these values were divided by $E_{280}^{1\text{cm}}$ values, units/ml directly approximated units/mg of

protein within acceptable limits for the products of all steps except for the final DEAE-Sephadex A-50 step and the following preparative polyacrylamide-gel electrophoresis run.

Activities were determined with a u.v. spectrophotometer co-ordinated with a Beckman model 1005 10-inch linear-log recorder. The cell compartment of the spectrophotometer was jacketed for temperature control by fluid circulation from a constant-temperature bath. A 1 cm light-path cuvette containing 3ml of substrate solution was placed in the compartment, and 5–20 μl of enzyme solution were pipetted directly into the substrate to start the reaction. The velocity was taken from the slope of the linear progress curves generated on the recorder.

Activities with acetylcholine and butyrylcholine were determined with Radiometer pH stats (Main, 1969).

Disc electrophoresis

Disc electrophoresis runs were made with a Savant Instruments apparatus, typically in $6\text{mm} \times 100\text{mm}$ glass tubing, by the standard system of Davis (1964). Staining for protein was with Coomassie Blue by the procedure of Chrambach *et al.* (1967). Preparative polyacrylamide-gel electrophoresis runs were made by the procedure of Main *et al.* (1972) by using a Buchler Instruments 'Poly-Prep' electrophoresis apparatus with an annular gel compartment area of 15.8cm^2 .

Determination of cholinesterase stability at pH 3.0

The pH was lowered to 3.0 ± 0.1 , as measured with a Beckman digital pH meter, by the addition of $0.5 \pm 0.02\text{ml}$ of 2.5M - H_2SO_4 to 25ml of serum or to 25ml of 33%-satd. $(\text{NH}_4)_2\text{SO}_4$ serum supernatant. The acid was added either slowly over a period of 30min, or rapidly in 2min. In the presence of $(\text{NH}_4)_2\text{SO}_4$, the rate of addition made little difference and timing was started when the solution reached pH 3.0. In the absence of $(\text{NH}_4)_2\text{SO}_4$, slow addition resulted in an apparent increased rate of inactivation when timing began at pH 3.0, compared with results obtained after rapid addition of the acid. The increase was due to inactivation during the slow addition of acid. The acid was therefore added rapidly, in 2min, for studies in the absence of $(\text{NH}_4)_2\text{SO}_4$. Timing began when the pH reached 3.0, and the solution was monitored continuously to ensure the pH remained at 3.0. Solutions of electrophoretically pure cholinesterase containing 6.5 activity units/ml in 0.1M -NaCl– 0.05M - NaHCO_3 were also lowered to pH 3.0 by rapid addition of 2.5M - H_2SO_4 .

After acidification, portions (approx. 1 ml) were withdrawn at progressive times and neutralized by mixing with 15–20 mg of powdered NaHCO_3 . The activity was measured 30 min after neutralizing. Failure to neutralize before assaying the activity resulted in non-linear progress curves.

For studies at 2°C , samples were left for 24 h at 2°C before the pH was lowered. It was essential to neutralize the time samples at 2°C . Raising the temperature even momentarily at pH 3.0 resulted in rapid loss of activity in samples without $(\text{NH}_4)_2\text{SO}_4$.

Stability of cholinesterase activity at pH 3

The study was restricted to conditions relevant to the acid step of the Strelitz (1944) method. The stability of the cholinesterase activity in serum at pH 3.0, both in the presence and absence of 33%-satd. $(\text{NH}_4)_2\text{SO}_4$, was determined at $2 \pm 1^\circ\text{C}$ and $24 \pm 1^\circ\text{C}$ (Figs. 1a and 1b). Also the stability at pH 3.0 of cholinesterase activity in serum was compared with that of electrophoretically pure cholinesterase at $2 \pm 1^\circ\text{C}$ and $24 \pm 1^\circ\text{C}$ (Fig. 1b).

Fig. 1. Effect of 33%-satd. $(\text{NH}_4)_2\text{SO}_4$ on the stability of cholinesterase at pH 3.0

(a) Comparison of the stability at $\text{pH } 3.0 \pm 0.1$ of cholinesterase activity in serum at $24 \pm 1^\circ\text{C}$ (●) and at $2 \pm 1^\circ\text{C}$ (○) and of cholinesterase activity at $\text{pH } 3.0 \pm 0.1$ in a serum supernatant, 33% saturated with respect to $(\text{NH}_4)_2\text{SO}_4$ at $24 \pm 1^\circ\text{C}$ (■) and at $2 \pm 1^\circ\text{C}$ (□). (b) Comparison of the stability at $\text{pH } 3.0 \pm 0.1$ of the activity of electrophoretically pure cholinesterase at $24 \pm 1^\circ\text{C}$ (○) and $2 \pm 1^\circ\text{C}$ (□) with the activity of cholinesterase in serum at $24 \pm 1^\circ\text{C}$ (●) and at $2 \pm 1^\circ\text{C}$ (■). The activity remaining was calculated as a percentage of the activity measured at neutral pH value, before it was adjusted to 3.0. Timing was started when the pH reached 3.0 ± 0.1 . About 2 min were required to lower the pH in the absence of $(\text{NH}_4)_2\text{SO}_4$. In the presence of $(\text{NH}_4)_2\text{SO}_4$ the pH was usually lowered to 3.0 from neutrality in about 30 min. However, lowering it in a shorter time did not affect the results. The time scale in Fig. 1(b) is expanded 12 times over that in Fig. 1(a) to show more clearly the course of inactivation at $24 \pm 1^\circ\text{C}$ in the absence of $(\text{NH}_4)_2\text{SO}_4$.

At pH3.0 and 24°C, 50% of the activity in serum was lost in about 25 min (Fig. 1b). In the presence of 33%-satd. $(\text{NH}_4)_2\text{SO}_4$, no significant loss had occurred after 2h, whereas 83% of the activity remained after 30h at pH3.0 and 24°C (Fig. 1a). This concentration of $(\text{NH}_4)_2\text{SO}_4$ appeared to slow the rate of destruction by a factor of about 1000 at 24°C.

At 2°C no significant loss of activity at pH3.0 had occurred after 26h in the presence of 33%-satd. $(\text{NH}_4)_2\text{SO}_4$, whereas in its absence 50% of the activity was lost after 19h. Clearly the 33%-satd. $(\text{NH}_4)_2\text{SO}_4$ appeared to offer almost complete protection for purification purposes at both 24°C and 2°C.

In the absence of $(\text{NH}_4)_2\text{SO}_4$, activity was rapidly lost at 24°C. Lowering the temperature to 2°C slowed the rate of loss by a factor of about 40 when the 50% loss point was used as an index. To achieve the full stabilizing effect of low temperatures, it was necessary to leave the enzyme at the low temperature for several hours before lowering the pH. Lowering the temperature rapidly, followed by acidification, resulted in a loss rate almost as great as that at 24°C.

$(\text{NH}_4)_2\text{SO}_4$ at 33% saturation also stabilized the pure enzyme, but the effect appeared to be more complex than for the enzyme in the serum supernatant.

The rate of loss of activity in serum was closely similar to that of electrophoretically pure cholinesterase at both 24°C and 2°C (Fig. 1b), suggesting that the purification procedure had not altered the enzyme in this respect.

Purification of cholinesterase from serum

The procedure involved two principal stages. In Stage 1, the serum proteins were fractionated with $(\text{NH}_4)_2\text{SO}_4$. Stage 2 involved ion-exchange chromatography of the product from Stage 1 on three successive DEAE-Sephadex A-50 columns.

In Stage 1, the acid step was retained in principle, but the enzyme was not precipitated directly from the acid supernatant as in the Strelitz (1944) procedure. Instead the acid supernatant was immediately neutralized after removal of the precipitate. A further precipitation of contaminating proteins was then effected by increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration from 33% to 54% saturation. The cholinesterase remained in solution. If not removed, the proteins precipitated by this step greatly decreased the purification achieved by the following ion-exchange chromatography steps. Similarly, in the last step of Stage 1, the cholinesterase was precipitated at the lowest concentration of $(\text{NH}_4)_2\text{SO}_4$ consistent with high yields. In general the steps of Stage 1 were designed to yield a product which could effectively be processed by the steps of Stage 2.

The time at which the enzyme stood at pH3.0 was kept to within 2h. The stability study indicated that no significant destruction of the enzyme would then occur, either at room temperature (23°C) or in the cold (5°C). This was confirmed by analysing both the supernatant and the precipitate for activity after the acid step. The activity recovered was $96.5 \pm 0.7\%$ of that in the samples before acidification, indicating that no significant loss had occurred.

Although both the stability study and recovery on the acid step indicated no immediate loss of activity, it seemed possible that delayed destruction of the enzyme, triggered by the acid step, might have occurred. Since the enzyme at pH3 was 40 times more stable in the cold than at room temperature, the possibility of delayed destruction was explored carrying the acid step out at both 5° and at 23°C. If delayed destruction had occurred, it seemed probable that it would be reflected eventually by a significantly less-active final product when the acid step had been done at 23°C compared with the activity of the product after an acid step done at 5°C.

In a typical run, 20 litres of serum were processed to the end of Stage 2. The purifications and yields obtained on each step after the acid treatment at 5° and 23°C are given in Table 1.

Stage 1: fractionation with $(\text{NH}_4)_2\text{SO}_4$. About 5 litres of serum were treated through to the completion of Stage 1 in 1 day. A Lourdes refrigerated centrifuge equipped with a 3.3-litre capacity rotor ($r_{av.}$, 12.5cm) was used. $(\text{NH}_4)_2\text{SO}_4$ concentrations are given as percentage saturation in water at 25°C from the table of Green & Hughes (1955). Protein impurities were precipitated from serum in three successive steps. The cholinesterase was precipitated from the supernatant of the third step to yield the product (P4) of Stage 1.

Step 1: fractionation with 33.6%-satd. $(\text{NH}_4)_2\text{SO}_4$. Solid $(\text{NH}_4)_2\text{SO}_4$ (200g/l) was added slowly to serum in about 30min at 23°C with vigorous mechanical stirring. Stirring was continued for a further 30min and the precipitate was removed by centrifuging at 4200g for 30min at 20°C. The supernatant (S1) was retained.

Step 2: fractionation at pH3 in the presence of 33%-satd. $(\text{NH}_4)_2\text{SO}_4$ at either 5°C or 23°C. Supernatant S1 was left 12h at 5°C before adding the acid at this temperature. When acidification was done at 23°C, fraction S1 was treated immediately after step 1. Except for the temperature, the procedures were the same. H_2SO_4 (20 ± 1 ml of 2.5M- H_2SO_4 /l of fraction S1) was added slowly by running it down the side of the vessel containing the vigorously stirred solution. When the pH reached 3.0 as measured by a pH-meter, addition of acid was stopped. The acid was added in 40 ± 5 min, regardless of the volume treated. The precipitate was removed

Table 1. Comparison of the purifications and yields of cholinesterase characterizing the procedure when the acid step was done at 5°C and at 23°C

Purifications were based on horse serum with a specific activity of 0.04 unit/mg of protein. On the basis of 20 different lots of pooled serum, 1 litre of serum contained 3650 ± 90 activity units and 89 ± 5 g of protein. Purifications of the products from the $(\text{NH}_4)_2\text{SO}_4$ stage were based on protein determined by measuring E_{280} . Protein was determined by the method of Lowry *et al.* (1951) in the products from the ion-exchange chromatography stage. S, supernatant; P, precipitate. The procedure is given in the text. Values are averages from two or more runs.

Product	Purification		Overall yield (%)	
	5°C acid step	23°C acid step	5°C acid step	23°C acid step
$(\text{NH}_4)_2\text{SO}_4$ stage				
Serum	1	1	100	100
S1	2.1	2.1	91.4	91.4
S2	9.4	11.1	66.8	70
(Acid step)				
S3	11.9	27	56.4	64
P4	21	80	52.8	54
DEAE-Sephadex A-50 column stage				
Column 1	860	1820	41.6	37.8
Column 2	6160	7980	31.2	37.0
Column 3	18850	18940	23.4	31.8

by centrifugation for 30 min at 4200 *g* at 5° or 20°C, depending on the temperature of acidification. The supernatant (S2) was retained and immediately brought to pH 7.8 by addition of 8 ml of 5M-NaOH/l of fraction S2. Not more than 2 h elapsed between the time the pH was lowered to 3.0 and the time it was raised again to 7.8.

Step 3: fractionation with 54%-satd. $(\text{NH}_4)_2\text{SO}_4$. Solid $(\text{NH}_4)_2\text{SO}_4$ was added at room temperature to the neutralized fraction S2 (130 g/l) as in step 1. The precipitate was removed by centrifugation at 4200 *g* for 40 min at 20°C. The supernatant (S3) was retained.

Step 4: precipitation of cholinesterase. When the acid step was done at 5°C, 52 g of $(\text{NH}_4)_2\text{SO}_4$ were added at room temperature/litre of fraction S3, bringing the concentration to 62.5% saturation. When the acid step was done at 23°C, 69 g of $(\text{NH}_4)_2\text{SO}_4$ /litre of fraction S3 were added, bringing the final concentration to 64.5% saturation. When the $(\text{NH}_4)_2\text{SO}_4$ was in solution, stirring was stopped and the precipitate was allowed to form for 1 h. It was then centrifuged for 45 min at 5000 *g* and 20°C. The precipitate (P4) was retained and dissolved in several portions of 50mm-sodium phosphate buffer, pH 8, totalling about 75 ml/litre of serum treated initially. The dissolved precipitate was dialysed twice at 5°C, first overnight against 6 litres of distilled and deionized water and then for a day against 6 litres of 40mm-sodium phosphate buffer, pH 8, to bring the final salt concentration to about 50mm. The product was ready for the first ion-exchange chromatography step of Stage 2.

Stage 2: ion-exchange chromatography on DEAE-Sephadex A-50. A 50mm-sodium phosphate buffer, pH 8.0, was used throughout Stage 2. The DEAE Sephadex A-50 was swollen in it, the columns and applied samples were equilibrated with it and the eluants and rinses contained it. Cholinesterase in this buffer adhered well to DEAE-Sephadex and column flow rates were readily maintained. The column dimensions given were measured after packing and elution of the column with this buffer at a head of 12 cm.

The linear gradients were developed by using a modification of the gradient apparatus described by Parr (1954) and were calculated from the equations of Peterson & Sober (1962). The apparatus consisted of two glass bottles (bottle 1 and bottle 2) of equal capacity and cross-sectional area. Each bottle contained two openings, one at the top and one at the bottom. The bottles were connected through the bottom openings so that the contents of bottle 1 flowed into bottle 2 as the contents of bottle 2 were drawn off through the DEAE-Sephadex column. The liquid head in bottle 2 was kept constant, and the liquid levels in bottles 1 and 2 were kept equal throughout delivery. The contents of bottle 2 were stirred continuously. The gradients developed were tested and found to be linear.

Although the products from acid treatment at 5°C and 23°C were of quite different degrees of purification (Table 1), the Stage 2 treatment was the same for both.

Column 1: treatment with choline chloride elution. The Stage 1 products applied to the first column were

Fig. 2. Selective elution of cholinesterase from the first DEAE-Sephadex A-50 column with a linear choline chloride gradient

(a) Activity (○) and protein (●) elution profiles characterizing a run made with a sample from the $(\text{NH}_4)_2\text{SO}_4$ stage in which the acid step was performed at 23°C. (b) Comparable profiles characterizing a sample prepared by way of an acid step at 5°C.

each derived from 5 litres of serum and contained about 9500 units in 350ml. When the acid step was done at 5°C, the sample contained about 9g of protein; and when it was at 23°C, 3.3g of protein were present. A column (26cm × 5cm diameter) with a bed volume of 510ml was used. The sample was applied at a rate of about 100ml/h and was rinsed at the same rate with 1500ml of 30mM-choline chloride in 50mM-sodium phosphate buffer, pH 8.0. The rinse removed 25–40% of the protein applied, but did not remove activity. The column was eluted with a linear choline chloride gradient of 0.29mM/ml. The gradient was developed by placing 500ml of 640mM-choline chloride in 50mM-sodium phosphate, pH 8.0, into bottle 1 and 1000ml of 30mM-choline chloride in 50mM-sodium phosphate, pH 8.0, into bottle 2. Typical elution profiles of the Stage 1 products from

acid steps at 23° and 5°C are compared in Figs. 2(a) and 2(b) respectively.

The cholinesterase activity reached a peak at 75mM-choline chloride (Figs. 2a and 2b). When NaCl replaced choline chloride as the eluent under otherwise identical conditions, the activity reached a peak at 142mM-NaCl; and the purification achieved was decreased by a factor of 5, indicating that choline chloride was a selective eluent for cholinesterase.

The pooled eluents (200–250ml) were dialysed overnight at 5°C against 6 litres of 45mM-sodium phosphate buffer, pH 8.0.

Column 2: treatment with NaCl elution. The combined products from two column 1 runs containing about 13000 units in 500ml were applied to the second column. The sample was derived from 10 litres

EXPLANATION OF PLATE I

Evidence of purity as indicated by the typical disc-gel electrophoresis patterns obtained with the cholinesterase product from preparative polyacrylamide-gel electrophoresis prepared in this example by way of an acid step at 5°C

Reading from the left, gels 1, 2, 4 and 5 were stained for protein with Coomassie Blue. Gels 3 and 6 were stained for activity with indoxyl acetate substrate. Similar activity patterns were obtained when butyrylthiocholine was the substrate. The amount of protein applied to each gel was as follows: gel 1, 60 μ g; gels 2 and 3, 40 μ g; gel 4, 20 μ g; gels 5 and 6, 10 μ g. The samples were run on 7% (w/v) acrylamide gels in tubes (6 mm $\times$ 100 mm) by the standard method of Davis (1964).

EXPLANATION OF PLATE 2

Evidence of purity as indicated by the activity and elution profiles of final products

(a) Evidence of purity as indicated by the activity (○) and protein (●) elution profiles obtained when the cholinesterase product from preparative polyacrylamide-gel electrophoresis was applied to a column (1.1 cm $\times$ 6 cm) of DEAE-Sephadex A-50 and eluted with 150 mM-choline chloride in 50 mM-sodium phosphate, pH 8. Further details are in the text. (b) Activity (○) and protein (●) elution profiles characterizing the product from a final (fifth) successive column of DEAE-Sephadex A-50. The run was an attempt to avoid preparative gel electrophoresis of the Stage 2 product by subjecting it to two additional ion-exchange chromatography column steps to remove the remaining impurities. The pooled product from the run was analysed by analytical disc-gel electrophoresis on 7% (w/v) gels. The inset picture shows a typical gel pattern obtained after staining for protein with Coomassie Blue. About 40 μg of sample were applied. A comparable pattern obtained with the product from preparative gel electrophoresis is shown in the inset of (a).

of serum and contained about 420mg of protein when its preparation had included an acid step at 5°C and 160mg when the acid step had been done at 23°C. A column (9cm × 2.5cm diameter) with a bed volume of 45ml was used.

The second column step depended primarily on a rinse with 250ml of 77.5 mM-NaCl in 50mM-sodium phosphate buffer, pH8.0, at the rate of 25ml/h. The rinse washed 70–85% of the protein impurities from the column. The column was eluted with a linear NaCl gradient of 0.32mm/ml and the eluent was collected in fractions of volume 5ml. The gradient was developed by placing 350ml of 300mM-NaCl in 50mM-sodium phosphate, pH8.0, in bottle 1 and 350ml of 77.5mM-NaCl in 50mM-sodium phosphate in bottle 2. The pooled eluent containing the activity (90–150ml) was dialysed overnight at 5°C in 6 litres of 45mM-sodium phosphate, pH8.0.

Column 3: treatment with choline elution. The combined products from two column 2 treatments, derived from 20 litres of serum and containing about 21000units and 100mg of protein, were applied to the third column. A column (7cm × 2.2cm diameter) with a bed volume of 30ml was used. The sample was applied at the rate of about 20ml/h and was rinsed with 100ml of 50mM-sodium phosphate buffer, pH8.0. The column was eluted with a linear choline chloride gradient (0.31mm/ml) and was collected in 5ml fractions. The gradient was developed by placing 400ml of 250mM-choline chloride in 50mM-sodium phosphate buffer, pH8.0, into bottle 1 and 400ml of 50mM-sodium phosphate, pH8.0, into bottle 2. The pooled eluent (80–90ml) containing the activity was the product from Stage 2. It was 85–90% cholinesterase and was purified about 19000-fold with respect to serum proteins. The yield varied depending on the temperature of the acid step (Table 1). The products weighed 20 ± 4 mg (dry wt., two runs) when the acid step was at 5°C and 28 ± 1 mg (dry wt., two runs) when the acid step was at 23°C.

Preparation of electrophoretically pure cholinesterase. To obtain electrophoretically pure cholinesterase, the 10–15% of impurities remaining in the product from Stage 2 were removed by preparative polyacrylamide-gel electrophoresis by the procedure of Main *et al.* (1972). The Stage 2 product was concentrated for electrophoresis by precipitation from 70%-satd. $(\text{NH}_4)_2\text{SO}_4$. After centrifugation the precipitate was dissolved in the electrophoresis sample buffer and made to a final volume of about 7ml. Each run was made with 20–25mg of protein and one pass was sufficient to produce electrophoretically pure material (Plate 1).

Running the electrophoresis apparatus before applying the sample did not remove the gel contaminants which were eluted with the cholinesterase (Main *et al.*, 1972). The gel contaminants were

therefore removed by applying the pooled product from the electrophoresis run (40–50ml) to a thoroughly washed column (1.1cm × 6cm) of DEAE-Sephadex A-50 at 4°C by using the same buffers and procedures as in the previous column steps. The contaminants were rinsed off with 200ml of 50mM-sodium phosphate, pH8.0. The cholinesterase was then eluted with 150mM-choline chloride in the same buffer. A typical elution profile is shown in Plate 2(a). The yield was 15–18mg.

An attempt was made to avoid preparative electrophoresis by subjecting the Stage 2 product, associated with an acid step at 5°C, to two additional column runs eluted with linear choline chloride gradients. The attempt was partially successful in the sense that the specific gravity of the product from this treatment was the same as that from preparative electrophoresis (Table 2). However, analytical disc-gel electrophoresis indicated that traces of impurity remained (Plate 2b).

Characterization of the purified cholinesterase preparation

Some properties of purified cholinesterase. The pooled product from the DEAE-Sephadex column (Plate 2) was subjected to exhaustive dialysis against two changes of 3 litres each of distilled, deionized water at 5°C. Unlike eel acetylcholinesterase (Leuzinger *et al.*, 1968), the horse cholinesterase appeared to be reasonably soluble in water (>0.45 mg/ml); and also it was stable. The small loss of activity appeared to be reversible (Table 2).

The possibility that cholinesterase could be obtained as a fully active pure salt-free dry powder was therefore examined by subjecting the non-diffusible material to freeze-drying from an ambient temperature of 5°C. The freeze-dried powder was dried to constant weight over P_2O_5 and then redissolved in 200mM-Tris-HCl buffer, pH8.0. None of the activity had been lost (Table 2), indicating that pure cholinesterase could be stored and handled as a pure, dry powder.

The possibility that final purification by preparative gel electrophoresis might have resulted in partial inactivation of cholinesterase was examined by comparing the specific activities of the highly purified material (+95% cholinesterase) obtained from the additional column runs (Plate 2) with that of the preparative electrophoresis products. The results (Table 2) indicated that the activity had not been affected by preparative gel electrophoresis.

The $E_{280}^{\text{mg/ml}}$ of cholinesterase based on dry-weight determination averaged 1.36 ± 0.07 for the preparations in Table 2. The $E_{280}^{\text{mg/ml}}$ value based on dry weight of the cholinesterase produced previously (Main *et al.*, 1972) was 1.5, whereas the value reported by Jansz & Cohen (1962) and used by Lee & Harpst (1971) was 2.5. The $E_{280}^{\text{mg/ml}}$ of eel acetylcholinesterase was reported to be 1.61 by Leuzinger *et al.* (1968).

Table 2. *Stability and some properties of highly purified cholinesterase subjected to preparative polyacrylamide-gel electrophoresis, exhaustive dialysis against distilled water and freeze-drying*

Specific activity is given as units/min per mg dry wt. at 25°C. Purification was based on specific activity value of 0.04 for horse serum. The products from the first and second freeze-drying steps weighed 15.7 and 11.7 mg respectively, after drying to constant weight at 4°C over P₂O₅.

Product from	Treatment	Solvent	Specific activity	Purification	$E_{280}^{mg/ml}$	E_{280}/E_{260}
Final DEAE A-50 chromatography step	None (control)	Cholinesterase in 150 mM-choline chloride-50 mM-sodium phosphate, pH 8.0	760	19000	1.304	1.772
Preparative electrophoresis	Preparative electrophoresis and ion-exchange on DEAE A-50 to remove gel contaminant	Cholinesterase in 50 mM-Tris-HCl buffer, pH 8.0	762	19050	1.298	1.788
Preparative electrophoresis after dialysis	Dialysis against two changes (3 litres each) of water at 4°C	Cholinesterase in water at 4°C	698	17440	1.347	1.698
Preparative electrophoresis after freeze-drying	Freeze-drying to anhydrous powder at ambient temperature of 4°C	Cholinesterase powder dissolved in 200 mM-Tris-HCl buffer, pH 8.0	778	19460	1.477	1.529
Preparative electrophoresis	Second dialysis and freeze-drying to anhydrous powder	Cholinesterase powder dissolved in 200 mM-Tris-HCl buffer, pH 8.0	709	17725	1.358	1.203

Evidence of purity. Purity of the final products was judged primarily by the patterns obtained on disc gels, by the constancy of the specific activities across elution profiles and by the symmetry of the schlieren peaks observed during sedimentation-velocity runs on the analytical ultracentrifuge.

Typical disc-gel patterns characterizing the product from preparative electrophoresis are shown in Plate 1. The sample shown was obtained by the procedure in which the acid step was done at 5°C. Material obtained by way of an acid step at 23°C showed similar patterns. Staining for protein by Coomassie Blue revealed one very predominant band indicating that pure cholinesterase existed almost exclusively in one form during disc electrophoresis. Staining for activity with indoxyl acetate showed a heavy band corresponding to the protein band; but two, more mobile, bands appeared below the main activity band. The gels were also stained lightly for activity above the main band. Two very faint protein bands appeared above the main band on the most heavily loaded gels (Plate 1). (The band nearest the top is an optical artifact and appears with the same intensity on all the gels stained for protein, regardless of their loading.) The additional activity bands were observed with earlier preparations (Main *et al.*, 1972), and the material in them was shown to be from the predominant form of cholinesterase. Moreover, studies of shoulder material from preparative electrophoresis runs suggest that concentrated solutions of cholinesterase tend to aggregate. This may explain the faint bands of less-mobile material observed above the main protein band on the more heavily loaded gels.

Quantitative estimates made by comparing the bands on gels to which a wide range of cholinesterase loadings had been applied (0.1–80 µg) indicated that the minor bands contained about 0.1 µg of protein or less when 60 µg had been applied, indicating that the cholinesterase was at least 99% pure.

A typical elution profile of the DEAE-Sephadex column run which served to remove the gel contaminants is shown in Plate 2(a). The peak tube (5 ml) contained 84% of the activity (9605 units). The specific activities on both sides of the peak were within 5% of the peak material, and the variation was of a random nature indicating a high degree of purity. The comparable elution profiles of material obtained by way of an acid step at 23°C were also characterized by constant specific activities across the entire peak.

By way of comparison, the elution profile from the last of the additional DEAE-Sephadex column treatments is shown in Plate 2(b). Although the protein and activity peaks appeared to be closely superimposed, the specific activities of the descending or right-hand side of the peak were consistently lower than those on the peak and on the ascending side. The inset acrylamide-gel pattern indicated, nevertheless, that the product was about 95% pure.

Sedimentation velocity runs were made with the products from preparative gel electrophoresis associated with acid steps at both 5°C and 23°C. The runs were made on a Beckman model E analytical ultracentrifuge at 20°C by using a double-sector cell with sapphire windows and an AN-D rotor. The samples were equilibrated by dialysis against a 150 mM-sodium phosphate buffer, pH 7.6. Since the purpose

Fig. 3. Schlieren peak characterizing the cholinesterase product from preparative gel electrophoresis photographed 40 min after attaining speed during a sedimentation-velocity run made at 187069 g (r_{av} , 6.5 cm) on a Beckman model E ultracentrifuge

The sample (6.3 mg of protein/ml) was in 150 mM-sodium phosphate, pH 7.6. See the text for further details.

here was to test for homogeneity, high protein concentrations were used. The concentration of the product associated with the acid step at 23°C was 6.3 mg/ml, whereas that associated with the acid step at 5°C was 11.2 mg/ml. Only one symmetrical schlieren peak developed with each product. There was no evidence of material in the shoulders. The concentration of 11.2 mg/ml was so high that an optical distortion in the form of a bar down the peak line appeared on every plate. The same type of concentration distortion appeared as the peak first developed in the run made with the material from the acid step at 23°C. As time progressed the boundary diffused and the distortion disappeared. Photographs were taken at 4 min intervals with 40 s exposures. The picture taken 40 min after speed had been attained midway through the run is shown in Fig. 3. The symmetry of the peak developed and the complete absence of any shoulder material over the entire run, even at this high concentration, indicated that the product was essentially homogeneous. This was true also of the material from the acid step at 5°C.

The run was made at 187069 g (r_{av} , 6.5 cm). The $s_{20,w}$ value was 10.55×10^{-13} S. Runs made at lower concentrations and extrapolated to zero concentration gave an $s_{20,w}^0$ value of 11.1×10^{-13} S.

Comparison of specific activities of electrophoretically pure cholinesterase preparations. The specific

Table 3. Comparison of the specific activities of electrophoretically pure cholinesterase prepared from (A) partially purified commercial preparation, (B) from serum by the procedure associated with an acid step at 5°C, (C) from serum by the procedure associated with an acid step at 23°C, and (D) cholinesterase in untreated serum

The substrate concentrations were 1 mM. The specific activities were μ mol of substrate hydrolysed/min per mg dry wt. at 25°C, pH 8. The thiocholine ester activities were measured by the method of Ellman *et al.* (1961). The choline ester activities were measured with pH-stats (Main, 1969).

Sample	Specific activity with			
	Butyrylcholine	Butyrylthiocholine	Acetylcholine	Acetylthiocholine
A	919	506	291	300
B	1380	762	382	381
C	1431	734	—	—
D	0.085	0.041	0.024	0.022

activities of three electrophoretically pure preparations are compared in Table 3. The specific activities of the preparations obtained by the present procedure by using an acid step at either 5°C or 23°C agreed closely when compared on the basis of the two substrates butyrylcholine and butyrylthiocholine. This close agreement suggested that the acid step did not trigger a delayed destruction of active sites and indicated that these products were fully active.

In contrast, the specific activities of the pure preparations obtained when a partially purified commercial preparation was used as the starting material (Main *et al.*, 1972) were significantly lower than those of the present preparations, based on comparisons with four substrates. When the comparison was based on the two butyryl esters, the specific activities of the present products were 50% higher than those of the pure preparations obtained from the commercial material. Similarly when the specific activities were compared on the basis of the acetyl esters, the preparation from the acid step at 5°C was 29% higher. The averaged specific activities of the present preparations were then about 40% higher.

The substrate concentration of 1 mM was selected for convenience in connexion with the use of the method of Ellman *et al.* (1961). K_m and V values could not be determined by using the standard Michaelis equation because the kinetics of the substrate reaction were too complex. As shown in Table 3, the rate of hydrolysis of butyrylthiocholine determined by the method of Ellman *et al.* (1961) was 51.3% that of the butyrylcholine rate determined by pH-stat (734 compared with 1431 μ mol/min per mg dry wt.). Similar ratios were observed with the other products examined, including serum. To

determine if this reflected a fundamental difference in the substrate specificity of cholinesterase, the rate of hydrolysis of 1 mM-butyrylthiocholine by the product obtained by way of an acid step at 23°C was determined by pH-stat under comparable conditions, and was 1528 μ mol/min per mg dry wt. or 106.8% that of the rate characterizing the hydrolysis of butyrylcholine. When determined by the same method (by pH-stat), butyrylthiocholine was then hydrolysed at about the same rate as butyrylcholine. The relatively low rate must therefore have followed from the conditions employed in the method of Ellman *et al.* (1961) and did not reflect a fundamental difference in the substrate specificity of cholinesterase.

The method of Ellman *et al.* (1961) was tested on numerous occasions and was found to be highly reproducible.

Preliminary titrations with di-isopropyl phosphorofluoridate of the pure preparations associated with an acid step at 5°C gave a weight per active site of 101 300 $\pm$ 2900. By comparison, the weight per active site of the pure preparations obtained from commercial starting material was 143 500 $\pm$ 2500 (Main *et al.*, 1972) or about 40% higher. This clearly suggested that the differences in specific activities could be attributed to a smaller number of active sites on the cholinesterase molecules obtained by the earlier procedure. The possibility that the lower number followed from partial destruction of active sites during preparation of the commercial product could not be

examined, since the details of the method were not known, apart from the fact that it was prepared by a modified Strelitz (1944) procedure (Worthington Enzyme Manual, 1972, p. 67). The step in the Strelitz (1944) procedure which could lead to partial destruction is the one which involved solution of the filter-cake containing the enzyme precipitated from the acid supernatant. The filter-cake is dissolved in water resulting in a solution which is quite acid, pH approx. 3.3, but the cholinesterase is no longer protected by high salt concentrations. Under these conditions significant loss of activity could occur within a few minutes. The extent of the loss would depend on how long the solution was left before the addition of the $(\text{NH}_4)_2\text{SO}_4$ called for in this step. It seemed unlikely that the subsequent gel filtration and preparative electrophoresis steps used in the earlier procedure to finally purify the enzyme would lead to partial destruction. However, this possibility was not finally ruled out.

Whatever the reason for the lower specific activity, the point of central significance was that the minimum number of active sites per molecule of cholinesterase now appeared to be greater than the value of 2 indicated for the earlier preparation (Main *et al.*, 1972). The number of active sites would depend on the weight per active site and on the molecular weight of cholinesterase. The earlier value of 2 was based on a molecular weight of 315 000 determined by gel filtration. Subsequently Lee &

Table 4. *Amino acid and carbohydrate composition of horse serum cholinesterase*

Amino acid residue and glucosamine values were determined on a Beckman model 118 amino acid analyser by the standard procedure. Values are for the μ g of each residue in 1 ml portions containing 230 μ g of cholinesterase. Recovery was 91.7%. For additional details see the text.

Amino acid or saccharide residue	Residue determined (μ g)	Composition by weight (%)	Mol %	Number of residues per particle wt. of 101 000
Histidine	3.22	1.52	1.36	6
Lysine	12.67	6.05	5.71	24
Arginine	12.28	5.63	4.54	19
Aspartic acid and asparagine	18.98	8.94	9.52	39
Threonine	10.47	5.05	5.99	24
Serine	9.53	4.35	6.31	26
Glutamic acid and glutamine	21.16	10.03	9.52	39
Proline	7.83	3.77	4.76	19
Glycine	6.61	3.17	6.70	27
Alanine	6.52	3.16	5.29	22
Half-cystine	2.56	1.13	1.45	6
Valine	8.92	4.13	5.20	21
Methionine	3.59	1.82	1.58	6
Isoleucine	6.44	3.14	3.29	13
Leucine	14.87	6.91	7.62	31
Tyrosine	8.52	4.07	3.01	12
Phenylalanine	12.54	6.13	4.93	20
Tryptophan	7.67	3.62	2.38	10
Glucosamine	18.33	8.75	5.38	22
Hexoses	11.46	5.40	4.08	17
N-Acetylneuraminic acid	6.84	3.23	1.36	6

Harpst (1973) reported a value of 440000 on the basis of sedimentation-equilibrium studies. Before a definitive estimate could be made it would, among other things, be necessary to establish the molecular weight of cholinesterase within closer limits. Moreover, experience with eel acetylcholinesterase suggests that determining the number of active sites by titration with irreversible organophosphate inhibitors may be more difficult than anticipated. For example Kremzner & Wilson (1964) reported four sites, whereas Leuzinger (1971) reported two sites for acetylcholinesterase preparations prepared by essentially the same procedure.

Amino acid and carbohydrate composition. The amino acid and carbohydrate composition of an electrophoretically pure sample of cholinesterase prepared by the procedure by using an acid step at 5°C is given in Table 4. The analysis indicated that cholinesterase was a glycoprotein consisting of 17.4% carbohydrate, including 3.2% *N*-acetylneuraminic acid. The amino acid residue and glucosamine values were determined on a Beckman model 118 amino acid analyser by the standard procedure. The amino acid residue values were corrected for destruction, if any, during acid hydrolysis by extrapolation of 22h and 72h hydrolysate values back to zero time by assuming first-order kinetics (Hirs *et al.*, 1954). Each value was the average obtained from duplicate hydrolysates. Tryptophan was determined by the method of Spies & Chambers (1949) after unfolding by the procedure of Harrison & Hofmann (1961). Glucosamine was determined on the amino acid analyser by using hydrolysates subjected to 4M-HCl for 6h at 110°C *in vacuo*. Hexoses were determined by two methods which were in good agreement: the orcinol-H₂SO₄ method described by Graham *et al.* (1970) and the anthrone-H₂SO₄ method described by Umbreit & Burris (1972). *N*-Acetylneuraminic acid was determined by the direct Ehrlich reaction by the procedure of Werner & Odin (1952).

Partial specific volume. The partial specific volume was determined with an Anton Paar Precision Density Meter (model DMA 02L). Electrophoretically pure cholinesterase prepared by way of the acid step at 5°C and dissolved in 200mM-Tris-HCl buffer, pH8.0, was used. The concentration was 11.5mg (dry wt.)/ml.

The partial specific volume of the cholinesterase was then 0.688 ± 0.04 ml/g. The partial specific volume of bovine serum albumin determined under the same conditions was 0.734 ml/g which agreed with literature values.

The partial specific volume calculated from the amino acid and carbohydrate content, by assuming that glucosamine was in the form of *N*-acetylglucosamine, was 0.712 ml/g. In a preliminary investigation a partial specific volume of 0.71 ml/g was determined

by the method of Edelstein & Schachman (1967) by using a preparation which contained not more than 10% of impurities. The values obtained were in reasonable agreement, the 0.688 value by the precision density meter being about 3% lower than the other two values. The average was 0.703 ± 0.013 ml/g. The substantial carbohydrate content of cholinesterase probably accounted for a relatively low value. A value of 0.78 ml/g for $\bar{v}$ was reported by Lee & Harpst (1973) for horse serum cholinesterase. Without carbohydrate and by assuming that one-half of the glutamic and aspartic acid residues were amides, the partial specific volume calculated from the amino acid analysis was 0.732 ml/g. By way of comparison a value of 0.731 ml/g was calculated from the amino acid composition of eel acetylcholinesterase by Leuzinger *et al.* (1969).

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