Purification and properties of butyrylcholinesterase from horse serum

ARTICLE *in* ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS · AUGUST 1971

Impact Factor: 3.02 · DOI: 10.1016/0003-9861(71)90009-9 · Source: PubMed

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 145, 55–63 (1971)

Purification and Properties of Butyrylcholinesterase from Horse Serum[1]

JAMES C. LEE AND JERRY A. HARPST[2]

Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Received November 9, 1970; accepted March 16, 1971

A procedure has been developed to obtain a purified form of horse serum butyrylcholinesterase (BuChE) from a partially fractionated commercial preparation. A high recovery (greater than 80%) of the activity is achieved in each step of the purification procedure. In all steps involving column chromatography the cholinesterase activity is confined to a single, symmetrical activity peak. A single component is observed in the analytical ultracentrifuge over a 250-fold range of protein concentration (4–1000 μg/ml); the sedimentation coefficient, $s^0_{20,w}$, is 11.5 ± 0.3S. There is no indication of molecular aggregation or dissociation. The purified material consists of a single migrating species upon polyacrylamide gel electrophoresis in gels with different pore sizes and at neutral and alkaline pH. Kinetic data indicate that the enzyme exhibits normal Michaelis-Menten behavior. K_m for acetylcholine at pH 7.5 and 25° is 1.6×10^{-3} M and the Hill coefficient is one. Mg^{2+} and Ca^{2+} exert a stimulatory effect on the enzyme activity. It is concluded that the purification procedures employed yield a homogeneous form of BuChE, which is not contaminated by other detectable proteins.

Recent investigations (1–5) suggest that horse serum cholinesterase (acyl choline acyl hydrolase, EC 3.1.1.8) occurs as isozymes or as aggregates of a single form. Although none of these studies (1–5) was performed with a highly purified enzyme preparation, the data imply that the protein has some kind of quaternary structure. Since horse serum cholinesterase appears to have properties necessary for studying protein–protein interactions, which are important in metabolic, regulatory, and other biological processes, work in this laboratory was undertaken in an effort to provide a more complete physicochemical characterization of the molecular aggregation or subunit structure of the enzyme.

This report is concerned with the purification and some chemical properties of a horse serum cholinesterase obtained from a crude preparation which is available commercially. The purified enzyme is identified as butyrylcholinesterase (BuChE)[3]. Sedimentation and electrophoresis data indicate that the purified horse serum BuChE is a single molecular form. No evidence of isozymes or tendency toward aggregation has been detected under the conditions studied.

EXPERIMENTAL PROCEDURE

Materials

Research grade acetylcholine chloride and phenyl acetate were purchased from Matheson, Coleman, and Bell. Butyrylcholine iodide, acetyl-β-methylcholine bromide, propionylthiocholine iodide, butyrylthiocholine iodide, benzoylcholine chloride, propionylcholine iodide, β-naphthylacetate, tetrazotized *o*-dianisidine (diazo blue-B), and glycine (ammonia-free) were obtained from Nutritional Biochemical Co. Triethylamine and

[1] This work was supported in part by Public Health Service Grants GM-12794 and GM-13791 from the National Institute of General Medical Sciences and by American Cancer Society Grant E-484 to JAH.

[2] Recipient of Public Health Service Research Career Development Award GM-14252 from the National Institute of General Medical Sciences.

[3] The abbreviations used are BuChE, butyrylcholinesterase; AChCl, acetylcholine chloride.

acetylthiocholine iodide were purchased from Aldrich Chemical Co. Analytical reagent grade potassium acid phthalate and sucrose were obtained from Mallinckrodt Chemical Works. Trizma Base and EDTA were purchased from Sigma Chemical Co. and ammonium persulfate from J. T. Baker Chemical Co. Column media were Munktell's cellulose powder obtained from LKB-Produckter, Sephadex G-25 and G-200 from Pharmacia Fine Chemicals Co., Inc., and Whatman DE-52 DEAE-cellulose powder from H. Reeve Angel and Co. Acrylamide, ethyl acetate, *N*-*N'*-methylenebisacrylamide, *N*,*N*,*N'*,*N'*-tetramethyl ethylenediamine and riboflavin were purchased from Eastman Kodak Co. All chemicals were used without further purification.

Methods

Enzyme Assay

All enzyme assays were performed by a titrimetric method in the Radiometer TTA 31 microtitration assembly, fitted with a waterjacket and sealed top and connected to a TTT 11 Titrator, PHM 25/SE pH meter, and Magnetic Valve MNV1 which controlled delivery of titrant. All titrations were carried out under a continuous stream of prepurified nitrogen at conditions specified by Jansz and Cohen (6). Titrants were 0.01 N NaOH, standardized against potassium acid phthalate, or 0.001 N NaOH, which was used for substrate concentrations below 3×10^{-4} M. Standard 1-ml assay solutions contained one-tenth of the reagents described by Jansz and Cohen (6), 0.2–0.3 unit of enzyme, and glass-distilled water, which was boiled and stored under CO_2-free conditions. Plots of initial velocity vs time and amount of enzyme were linear.

One unit of enzyme activity is taken as the enzyme required to hydrolyze 1 μmole of acetylcholine per minute under the standard assay conditions described. Specific activity is in units per mg protein.

Kinetics

All kinetic studies were made by measuring initial velocities under the conditions chosen for each experiment. Initial velocities obtained at a pH different from 7.5 were corrected for substrate hydrolysis at the pH of measurement. Relative activities toward a variety of substrates were determined either by comparing values of V_{max} or by comparing hydrolysis rates obtained under standard assay conditions, where the appropriate substrate was substituted for acetylcholine.

Protein Concentration

Protein concentration was determined from absorbance at 280 nm with the extinction coefficients recommended by Jansz and Cohen (6).

Enzyme Purification

Step I. Commercial preparation. Crude enzyme from pooled horse serum[4] was purchased from Worthington Biochemical Corp. (Freehold, N. J.). The enzyme was received as a frozen ammonium sulfate precipitate (70% saturated) of the active supernatant fraction obtained from the third stage of the Strelitz procedure (7).

Step II. Differential sedimentation. Approximately 2 g of the commercially available precipitate was dissolved in 30 ml of phosphate buffer and dialyzed for 24 hr (6). This and all following manipulations were carried out at 3–5°. The dialyzed solution was filtered through a Gelman Metricel GA-3 membrane and centrifuged as described by Jansz and Cohen (6). The final pellet was dissolved in approximately 10 ml of 0.044 M triethylamine–carbon dioxide buffer, pH 8.5, to give a protein concentration of approximately 60 mg/ml.

Step III. Zone electrophoresis. Column electrophoresis of the solution from Step II was carried out as reported by Jansz and Cohen (6) on a 3 × 30-cm cellulose column in an LKB 3340 C apparatus. Continuous electrophoresis was maintained for 42 hr at 30 mA. Fractions with specific activities greater than 100 were pooled.

Step IV. DEAE-cellulose chromatography. The pooled fractions (24 mg protein at 0.6 mg/ml) from Step III were applied to a DEAE column (1 × 40 cm) which was then washed with 1 col vol of the triethylamine buffer, made 0.05 N in sodium chloride. Elution was carried out with a linear gradient formed from 0.2 N NaCl-buffer and 0.05 N NaCl–buffer at a flow rate of 20 ml/hr. The salt concentration of a 5000 × dilution of each 2.0-ml fraction was estimated with a Barnstead Purity Meter, PM-50. Fractions with specific activities above 400 were pooled and concentrated to 4 mg/ml with dry Sephadex G-25 (coarse)[5] in a Gelman centrifugal filter holder (8).

[4] A. L. Baker, Worthington Biochemical Corp., private communication.

[5] Early attempts to concentrate the enzyme gave large losses of active material (greater than 50%) essentially as reported by Heilbronn (1). However, a high recovery of activity could be obtained by a single rinse of the Sephadex with approximately 2 ml of buffer per 5 ml of wet gel. There was no loss in specific activity. In later experiments it was found that no loss of active en-

Step V. Sephadex G-200 fractionation. The concentrated enzyme solution from Step IV was applied to a Sephadex G-200 column (1 × 30 cm), which was connected in series to two identical columns with Teflon tubing (o.d., 0.21 cm). Potassium phosphate buffer, 0.01 M, pH 7.0, with which the columns had been equilibrated and washed thoroughly, was used for elution at a flow rate of 12 ml/hr. One-milliliter fractions with specific activities greater than 800 were pooled and concentrated to 1 mg/ml with dry Sephadex, as described for Step IV.

Sedimentation Velocity

The purified enzyme in 0.01 M potassium phosphate, pH 7.0, was examined in a Beckman-Spinco Model E analytical ultracentrifuge. Aluminum-filled Epon, double sector, 12-mm cells were used for all sedimentation velocity studies at 60,000 rpm and 5°. Schlieren photographs were recorded on Kodak Metallographic plates and analyzed on a Gaertner microcomparator. For solutions with protein concentrations below 0.5 mg/ml the sedimentation was monitored with the Beckman split-beam photoelectric scanner at 280 nm in routine studies and 236 nm for very dilute solutions. Sedimentation velocities were determined from the midpoints of the boundaries traced by the scanner. Values for the viscosity and density of water were used to correct all sedimentation coefficients to standard conditions.

Polyacrylamide Gel Electrophoresis

Disc electrophoresis on 7.5% and 5% polyacrylamide gels was carried out on 15–25 μg of purified protein (sp act 800–850) at pH 8.9 (9) and pH 7.0 (10). Experiments were run at a current of 4 mA/tube and room temperature for 2–6 hr as required. The gels were stained for protein for at least 1 hr with 1% amido schwarz in 7.5% acetic acid and destained electrophoretically (9). Other gels run under the same conditions were tested for esterase activity with the β-naphthol staining method described by Gomori (11). After electrophoresis the gel was removed from its glass tubing, immersed in a solution of β-naphthyl acetate for 5 min, rinsed with glass-distilled water, and then immersed in a solution of tetrazotized *o*-dianisidine for 2–3 min. A resulting violet band indicates the presence of esterase. Coincidence of the protein and activity bands was tested by splitting some gels lengthwise with a stainless-steel wire, returning half the gel to its glass tube and staining by the normal procedure for protein, and staining the other half as described for activity. All gels were stored in 7.5% acetic acid.

zyme occurred as long as the concentration was limited to 5-fold. Under these conditions the procedure could be repeated as necessary to achieve the desired final concentration.

RESULTS

Purification

A summary of the purification of horse serum BuChE is given in Table I. The recovery per step shown in the table is defined as the ratio of the total enzyme units, available at the beginning of the step, to the total final units of activity, obtained from that particular step. Because of inactive contaminants, only the primary enzyme fractions, those pooled for analyses and carried into later steps, have been used to calculate yield of purified material. The yield is based on the total units in whole serum, which has been estimated for Step I in Table I from the recoveries in the first three stages (26%) described by Strelitz (7). The data in the table show a high recovery of total activity in each step. The apparent loss of active enzyme in the pooled fractions is due mainly to the use of only the primary fractions of highest specific activity.

In the zone electrophoresis of Step III maximum recovery of enzyme activity was achieved when the protein concentration employed was 60 mg/ml or higher. A single, symmetrical activity peak was obtained in all runs, essentially as reported by Jansz and Cohen (6). The peak contained more than 90% of the total initial enzyme activity. In an effort to detect other esterases, each fraction from the electrophoretic column was tested against acetylcholine, butyrylcholine, benzoylcholine, and phenyl acetate. The esterase activity toward all these substrates was confined to the single, active peak. Other experiments, in which electrophoresis proceeded only until the active fractions had moved half-way through the column, were run in order to locate faster-moving cholinesterase components. Within the limits of detection of the standard assay, no other active fractions were found.

In Step IV a single BuChE activity peak was observed when elution from the DEAE column was carried out with a continuous salt gradient as shown in Fig. 1. Total en-

TABLE I

PURIFICATION OF HORSE SERUM BuChE

Purification step	Total enzyme activity			Primary enzyme fractions			
	Initial (A) (units)	Final (B) (units)	Recovery per step (B/A) (%)	Activity (pooled) (units)	Protein (pooled) (mg)	Yield[a] (%)	Specific activity (units/mg prot.)
I $(NH_4)_2SO_4$ ppt.	—	—	—	6,250	1,430	26	4.8
II Differential sedimentation	6,250	6,250	100	6,250	402	26	16.0
III Zone electrophoresis	6,250	6,142	98	5,100	24	21	214.0
IV DEAE chromatography	5,100	4,220	83	2,750	4.8	11	570.0
V Sephadex G-200 fractionation	2,750	2,600	95	2,300	2.7	9	850.0

[a] The yield is adjusted to that expected from whole serum and is calculated from initial total enzyme activity and the activity of the primary (pooled) fractions. See text for additional explanation.

zyme activity in the single peak accounted for at least 80% of the units initially loaded onto the column. Somewhat higher recovery was attained with increased flow rates, indicating that some of the loss of BuChE was due to inactivation on the column. Since there are several reports (1, 2) which describe stepwise gradient elution of BuChE from DEAE-Sephadex columns, efforts were made to incorporate stepwise elution into Step IV. This technique often gave multiple activity peaks similar to those described by Zech and Engelhard (2), but they were quite clearly artifacts due to overloading the column or improper selection of eluants. Consequently the method of choice is the continuous gradient elution illustrated in Fig. 1.

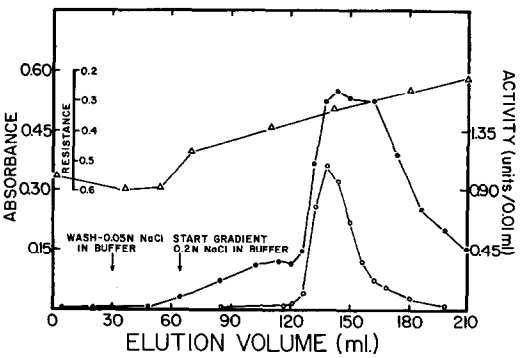

FIG. 1. DEAE-cellulose chromatography. Continuous gradient elution pattern for Step IV. NaCl gradient (△) is indicated by resistance measurements, made as described in the text. Absorbance (●) is at 280 nm. Enzyme activity (○) was measured against AChCl under standard assay conditions described in the text and is expressed as units/volume of enzyme solution.

Since a single column (1 × 30 cm) of Sephadex G-200 did not separate contaminating protein from BuChE in Step V, the combination of three columns in series was used to improve the resolution and give the elution profile in Fig. 2. The trailing peak has no BuChE activity and has been analyzed by ultracentrifugation, as described below. Because of the exclusion of BuChE and incomplete separation of the activity peak from the inactive, trailing material, it was essential to include in the final, pooled material only those fractions with specific activities greater than 800 (Fig. 2). Inclusion of any fraction with lower specific activity invariably gave a sample which showed a minor, slow-moving component upon ultracentrifugation.

Steps II–V result in a 150-fold purification of BuChE over that obtained for the commercially available ammonium sulfate precipitate from horse serum. The yield is 35–40% of the total activity in the commercial preparation. The yield with respect to whole serum, estimated in Table I as described above, is approximately 10%. The specific activity of this enzyme preparation is at least 5% greater than the highest value reported in the literature (6).

pH and Temperature Optima

The pH and temperature optima for BuChE were measured by changing the single variable under otherwise standard as-

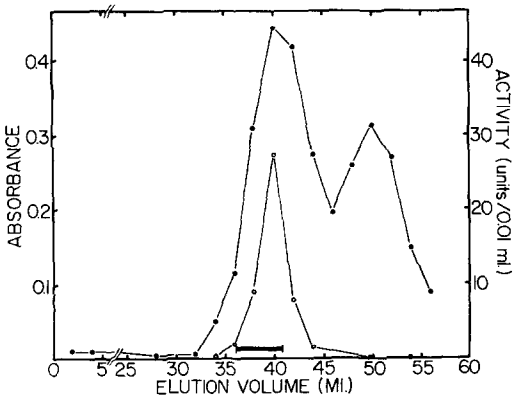

FIG. 2. Elution pattern from Sephadex G-200 fractionation with three columns in series, Step V. The symbols are the same as in Fig. 1. Fractions pooled to obtain the purified preparation are indicated by the solid bar.

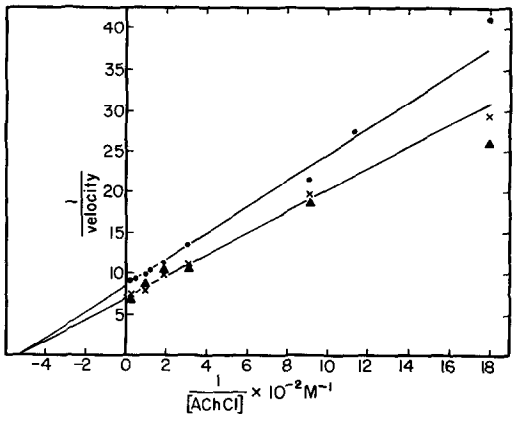

FIG. 3. Lineweaver–Burk plots. The initial velocity data are as follows: without additional metal ions (●); with 2.0×10^{-2} M Mg^{2+} (×); with 5.0×10^{-4} M Ca^{2+} (▲). There was 0.78 μg BuChE in the 1.0 ml of standard assay solution.

say conditions. The enzyme exhibited a broad pH activity profile with a pH optimum of 8.0, essentially as described by Jansz and Cohen (6). The temperature dependence was small around the optimum (36°), but activity dropped sharply below 32° and above 40°. The ratio of activity at 36° to that at 25° is 1.3. Both optima correspond to the physiological conditions for a mammalian enzyme.

Kinetics

The kinetics for hydrolysis of acetylcholine by BuChE at 25° and pH 7.5 were examined over a wide range of substrate concentrations (10^{-4}–10^{-1} M). No substrate inhibition was observed even at the highest substrate concentration studied as expected for BuChE (12). Linearity of the Lineweaver-Burk plot (13) over the entire range of substrate concentrations (Fig. 3) indicates that the purified BuChE follows normal Michaelis–Menten behavior. K_m for acetylcholine chloride under the conditions studied is $(1.6 \pm 0.3) \times 10^{-3}$ M, which is consistent with other results ($1–5 \times 10^{-3}$ M) reported on less pure enzyme preparations (14–16). The observation that the Hill coefficient is unity over a range of substrate concentrations between 1.1×10^{-2} M and 5.5×10^{-4} M indicates that BuChE exhibits no allosteric properties under the conditions for Fig. 3 (17).

Identification of cholinesterases by substrate activity tests has been described by Jansz and Cohen (6) and by Augustinsson (12). The results with purified BuChE and a number of substrates are shown in Table II, where values of K_m and V_{max}, as well as relative activities under standard assay conditions, are compared. Comparisons based on V_{max} do not differ significantly from relative activities measured under standard conditions. The enzyme was found to hydrolyze butyrylcholine at the highest rate (2.5 times faster than acetylcholine), had very little activity toward acetyl-β-methylcholine and ethyl acetate (Table II), and is not inhibited by high concentrations of acetylcholine. It has also been observed that the organophosphate, tetraethylpyrophosphate, at a concentration of 10^{-5} M completely inhibits activity, a characteristic of BuChE (3, 20). Since the kinetic results for the substrates shown in Table II essentially agree with the measurements of relative activities by other investigators (6, 12, 18, 19) the purified enzyme is a butyrylcholinesterase.

Both Ca^{2+} and Mg^{2+} exhibited a stimulatory effect on the purified BuChE. The possibility that the activity was affected solely by a change in ionic strength was ruled out, since sodium chloride decreased activity as its concentration increased. Added metal ions are not an absolute require-

TABLE II

KINETICS OF ESTER HYDROLYSIS BY BuChE

Substrate	$V_{max}/{}^{0}_{V_{MAX}}$[a] (%)	V/V^{0}[b] (%)	K_m (M × 10^3)
Acetylcholine chloride	100	100	1.6 ± 0.3
Propionylcholine iodide	152	168	1.0 ± 0.2
Butyrylcholine iodide	245	249	0.56 ± 0.06
Benzoylcholine chloride	15	14	0.23 ± 0.03
Phenyl acetate	7.3	6.6	4.0 ± 0.8
Acetylthiocholine iodide		143	
Propionylthiocholine iodide		229	
Butyrylthiocholine iodide		234	
AChCl + 10^{-3} M EDTA		85	
β-Naphthyl acetate		9.6	
Ethyl acetate		0.9	
Acetyl-β-methylcholine bromide		0	

[a] Values are expressed relative to the maximum velocity of AChCl (V^{0}_{max}) which is taken as 100.

[b] Values are expressed as velocity ratios (in percent) at a substate concentration of 5.5 × 10^{-2} M under standard assay conditions; V^{0} is the velocity with AChCl and V is that of the substrate indicated.

for the enzyme reaction, as indicated by a decrease of 15% in activity at all concentrations of EDTA between 10^{-5} and 10^{-2} M. The K_A of activation for Ca^{2+} was (1.5 ± 0.3) × 10^{-5} M and that for Mg^{2+} was (1.5 ± 0.3) × 10^{-2} M as shown in Fig. 4. The added metal ions had no effect on the K_m for AChCl (Fig. 3). Apparently, both ions activate the enzyme to the same extent (26% increase in V_{max}) but Ca^{2+} is more efficient, as indicated by its lower K_A- of activation. Since the graph of Fig. 4 is linear (13) and the Hill coefficient (17) is unity for each metal ion, there is binding of one kinetically significant metal ion per catalytic site.

Polyacrylamide Gel Electrophoresis

A single component was observed in both the 7.5 and 5% polyacrylamide gel disc electrophoresis of the purified BuChE under both alkaline (pH 8.9) and neutral (pH 7.0)

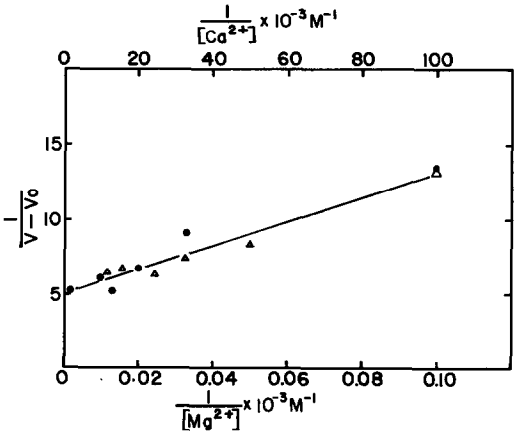

FIG. 4. Double-reciprocal plot for metal ion activation. All metal concentrations refer to ions added to the standard 1.0 ml assay solution. There was 0.78 μg of BuChE. Velocities are expressed as $(V-V_0)$, where V is μmole of AChCl hydrolyzed/min in the presence of the indicated amount of metals and V_0 is velocity without additional metals. Data are shown as follows: (●), with Ca^{2+}; (△), with Mg^{2+}.

conditions. Typical results are shown in Fig. 5. Furthermore, the gels have a single band which shows esterase activity, as illustrated by Fig. 5D. The band stained for activity was broadened, apparently due to diffusion of the dye between the time of staining and photographic recording. Since both gel concentrations give a single band (Fig. 5), it must not be an artifact of the sieving effect (21). The electrophoretic data clearly indicate that the purified BuChE is homogeneous.

Sedimentation Velocity

Analytical ultracentrifugation was used to monitor the purification through several steps. A BuChE preparation of intermediate purity and a specific activity of 200 (Step III) had three sedimenting components with values for $s_{20,w}$ of 3, 7, and 11S. Steps IV and V apparently remove all detectable amounts of the 3S component from the active material. The trailing and inactive protein obtained from the Sephadex G-200 fractionation step (Fig. 2) appeared upon centrifugation to be a single component with a value of $s_{20,w}$ = 7.3 at 0.8 mg/ml. Step V, there-

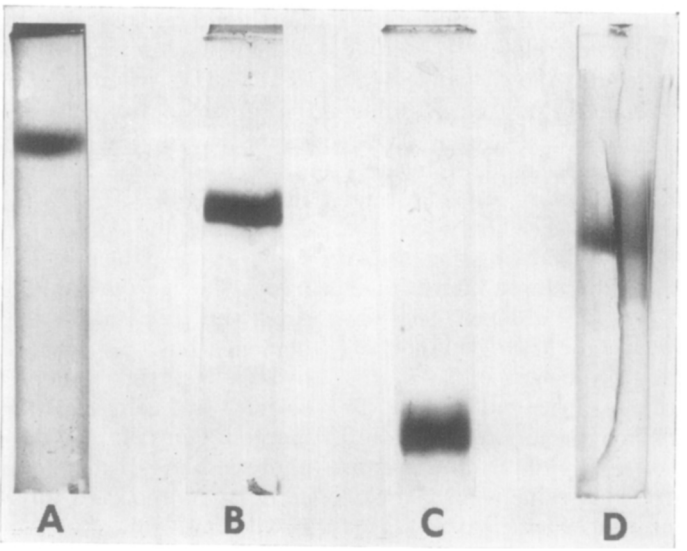

FIG. 5. Polyacrylamide gel electrophoresis of purified BuChE. A and B are electrophoretic patterns in 7.5% gel at pH 7.0 and 8.9, respectively. C is the pattern at pH 7.0 in 5.0% gel. D is the pattern at pH 8.9 in 5.0% gel split into two halves. The left half is stained for protein as for A, B, and C. The right half is stained for activity as described in the text. The amounts of protein utilized are 17 μg for A, C, and D and 25 μg for B.

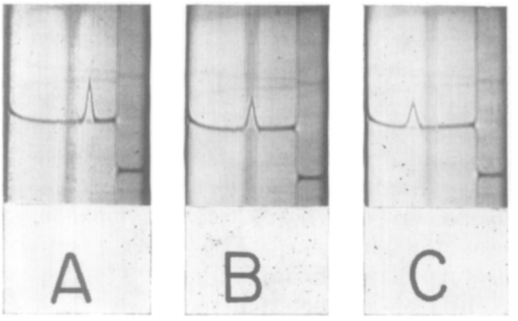

FIG. 6. Schlieren patterns from sedimentation of BuChE (sp act 820) at 0.92 mg/ml. A, B, and C were taken 24, 40, and 56 min, respectively, after reaching a speed of 60,000 rpm.

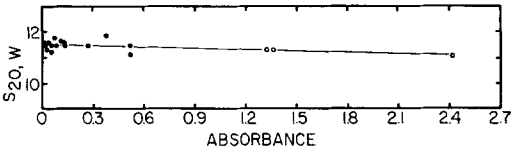

FIG. 7. Sedimentation coefficient versus absorbance at 280 nm. Data from both Schlieren (○) and photoelectric scanner (●) runs are included.

fore, separates the inactive 7S contaminant from the active material.

Active fractions from Step V (Fig. 2) which had specific activities greater than 800 (see above) always appeared as a single component in the ultracentrifuge, as shown in Fig. 6. The value $s^0_{20,w} = 11.5 \pm 0.3S$ was obtained for purified BuChE from the plot of $s_{20,w}$ vs absorbance at 280 nm, illustrated in Fig. 7. A normal linear dependence of $s_{20,w}$ on protein concentration was observed over a 250-fold range of concentration (4–1000 μg/ml). The small negative slope from the graph in Fig. 7 implies that BuChE is a compact molecule. Since there was no marked change in sedimentation coefficient at extreme dilution (4 μg/ml), BuChE undergoes no obvious molecular dissociation or aggregation under the conditions of the experiments.

DISCUSSIONS

The sedimentation properties observed during purification of the horse serum BuChE compare favorably with observations of other investigators. Zech and Engelhard (2) reported that one of their four fractions of horse serum cholinesterase showed three components (3, 6, and 8S)

in the ultracentrifuge. Sedimentation properties of this mixture closely resemble the three components (3, 7, and 11S) observed in the present study on a sample from Step III (Table I). As noted above, both the 3S and 7S components are inactive and removed in purification steps IV and V. The primary difference between the purified BuChE described in this paper and that described by Jansz and Cohen (6) appears to be the absence of their minor, contaminating 3.5S component. The sedimentation coefficient of 9.9S at 4 mg/ml reported by Jansz and Cohen (6) for their active enzyme compares favorably with the value of $s^0_{20,w} = 11.5$ obtained in the present study. This value is also similar to the sedimentation coefficient ($s_{20,w} = 9.7S$) reported by Tucci and Seifter (19) for porcine parotid BuChE, if approximate corrections for solvent and concentration are made.

A molecular weight for the purified horse serum BuChE was estimated to be in excess of 200,000 since the material is excluded by Sephadex G-200. Furthermore, a molecular weight between 300,000 and 400,000 can be expected from the sedimentation coefficient and the molecular weight determinations (368,000) for porcine parotid BuChE (19). Sedimentation equilibrium studies are in progress in this laboratory to determine the molecular weight of the BuChE from horse serum.

The kinetic measurements for horse serum BuChE can be compared to results on a purified cholinesterase obtained from porcine parotid glands by Tucci and Seifter (19). Both the purified horse serum and the porcine BuChE exhibit normal Michaelis–Menten behavior (Fig. 3). The values of K_m for various substrates correspond closely and there is no substrate inhibition by benzoylcholine, in contrast to the observation by Kalow on human serum cholinesterase (22). The porcine and horse enzymes have some obvious differences in the ratios of their maximum velocities. As illustrated in Table II the activity of horse serum BuChE is in the order butyryl- > propionyl- > acetylcholine, in contrast to the order butyryl- > acetyl- > propionylcholine as observed for the porcine BuChE. Furthermore horse serum BuChE has extremely low activity toward phenylacetate (Table II), whereas the porcine enzyme has much higher activity toward this substrate.

Electrophoretic behavior of horse serum BuChE both on the preparative column of Step III and on polyacrylamide gels after purification indicates that the protein is basic. The gel electrophoresis clearly indicates the preparation is electrophoretically homogeneous, a conclusion strengthened by the fact that the protein and esterase activity are coincident (Fig. 5). This is in marked contrast to the multiple electrophoretic bands which exhibit cholinesterase activity, as reported for whole horse serum by Oki *et al.* (3).

An interesting correlation, however, may be made between the purified BuChE and the results obtained by Oki *et al.* (3). The relative activities of the horse serum BuChE toward phenylacetate and benzoylcholine facilitate making a qualitative identification of the purified BuChE as the major cholinesterase component, C_4, observed in horse serum by the earlier workers (3). Although the relative activities toward benzoylcholine and phenylacetate cannot be compared strictly due to differences in assay conditions, the identification can be made on the basis that component C_4 was the only one which showed activity toward both substrates (3).

In view of the results of earlier investigations (1–5), it is surprising that the purified horse serum BuChE shows no trace of multiple molecular forms, aggregates, or isozymes. Although aggregation or dissociation of the preparation described in this paper is ruled out by tests of column eluants with a variety of substrates, the polyacrylamide gel electrophoresis, and by the sedimentation velocity measurements at extreme dilution, the possibility still exists that inhibition by organophosphates (5, 12, 18) causes structural changes in BuChE, thus affecting the kinetics of its reactions (5). Further work is necessary to determine if horse serum BuChE can be associated or dissociated by inhibitors, substrates, or denaturing agents.

The absence of isozymes, apparently

observed by numerous workers (1–4), is difficult to explain. It is clear that the purification procedures employed in the present investigation have removed all but a single protein component which exhibits cholinesterase activity. If the other forms of cholinesterase from horse serum were isozymes with similar physical properties, they could be expected to pass through at least the initial ammonium sulfate precipitations as a single component or as ones very difficult to separate. Since there are no detectable fractions containing cholinesterase activity, except for the single, active specie followed through Steps II–V of the purification procedures described in this paper, it appears that any other forms of cholinesterase may be unstable or may have been modified in the presence of ammonium sulfate. A further possibility is that the separable activities reported by other workers (1–4) are not isozymes but can be described more accurately in the words of Markert and Møller as "a family of enzymes the members of which have overlapping but distinct patterns of substrate specificity" (23). Further work on highly purified components is necessary to clarify the molecular basis for the multiple forms and/or isozymes of horse serum cholinesterases.

ACKNOWLEDGMENTS

The authors thank Drs. D. A. Goldthwait, K. E. Neet, and H. Z. Sable for their constructive criticism of the manuscript. We are also grateful to Dr. R. E. Barden for his helpful comments on the presentation of kinetic data.

REFERENCES

1. Heilbronn, E., *Biochim. Biophys. Acta* **58,** 222 (1962).
2. Zech, R., and Engelhard, H., *Biochem. Z.* **343,** 86 (1965).
3. Oki, Y., Oliver, W. T., and Funnell, H. S., *Can. J. Physiol. Pharmacol.* **43,** 147 (1965).
4. Reiner, E., Seuferth, W., and Hardegg, W., *Nature London* **205,** 1110 (1965).
5. Main, A. R., *J. Biol. Chem.* **244,** 829 (1969).
6. Jansz, H. S., and Cohen, J. A., *Biochim. Biophys. Acta* **56,** 531 (1962).
7. Strelitz, F., *Biochem. J.* **38,** 86 (1944).
8. Flodin, P., Gelotte, B., and Porath, J., *Nature London* **188,** 493 (1960).
9. Davis, B. J., *Ann. N. Y. Acad. Sci.* **121,** 404 (1964).
10. Williams, D. E., and Reisfeld, R. A., *Ann. N. Y. Acad. Sci.* **121,** 373 (1964).
11. Gomori, G., "Microscopic Histochemistry", p. 206. Univ. of Chicago Press, Chicago, Illinois (1952).
12. Augustinsson, K. B., *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd Ed., Vol. **4,** p. 521. Academic Press, New York (1960).
13. Lineweaver, H., and Burk, D., *J. Amer. Chem. Soc.* **56,** 658 (1934).
14. Yakovlev, V. A., *Biokhimiya* **31,** 258 (1966).
15. Brestkin, A. P., and Brik, I. L., *Biokhimiya* **32,** 3 (1967).
16. Kato, G., *Mol. Pharmacol.* **4,** 640 (1968).
17. Atkinson, D. E., *Ann. Rev. Biochem.* **35,** 85 (1966).
18. Oosterbaan, R. A., and Jansz, H. S., *in* "Comprehensive Biochemistry" (M. Florkin and E. H. Stotz, eds.), Vol. **16,** p. 1. Elsevier, New York (1965).
19. Tucci, A. F., and Seifter, S., *J. Biol. Chem.* **244,** 841 (1969).
20. Augustinsson, K. B., *Acta Chem. Scand.* **13,** 571 (1959).
21. Ornstein, L., *Ann. N. Y. Acad. Sci.* **121,** 321 (1964).
22. Kalow, W., *Can. J. Physiol. Pharmacol.* **42,** 161 (1964).
23. Markert, C. L., and Møller, F., *Proc. Nat. Acad. Sci. U.S.A.* **45,** 753 (1959).