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Composition and Molecular Weights of Butyrylcholinesterase from Horse Serum[1]

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The physical and chemical characterization of horse serum butyrylcholinesterase has been extended. The results show that the enzyme is a glycoprotein containing about 20% carbohydrate by weight. Mannose, glucosamine, galactose, and sialic acid are the sugar residues found. The extinction coefficient of butyrylcholinesterase, $E^{1\%}_{1\,cm}$ at 280 nm, was found to be 15.2 ± 0.3 by dry weight determination. The molecular weight of the protein in dilute phosphate buffer was determined to be (31.7 ± 1.2) × 10^4 by high speed equilibrium sedimentation with a redetermined partial specific volume of 0.723 ± 0.003 ml/g. Subunit molecular weights for the dissociated protein were found to be (7.9 ± 0.4) × 10^4 and (8.1 ± 0.1) × 10^4, respectively, in guanidine hydrochloride and in a solution at pH 11.8. The subunit molecular weight was also estimated to be (8.8 ± 0.2) × 10^4 by analytical sodium dodecyl sulfate-gel electrophoresis. This apparently higher subunit molecular weight from dodecyl sulfate gels is expected for glycoproteins containing significant amounts of carbohydrate. No free sulfhydryl group was detected, even though there are six half-cystines in each subunit. Therefore, it seems likely that there are three pairs of disulfide bonds per subunit. The available data indicate that native butyrylcholinesterase is a tetrameric glycoprotein consisting of subunits of equal molecular weight.

Previous work in this laboratory showed that butyrylcholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) from horse serum could be obtained in a purified form, which was homogeneous and stable (1). It was also shown by dissociation in Gdn·HCl[6] and at high pH that the native enzyme consisted of subunits, but in 0.01 M potassium phosphate, pH 7.0, the native enzyme did not dissociate or aggregate (2). Molecular weights for BtChE from horse serum have been reported previously by Lee and Harpst (2) and Main *et al.* (3), but the results do not agree. BtChE was shown to have a native molecular weight of 315,000 by analytical gel filtration and a subunit molecular weight of 77,300 by SDS-gel electrophoresis (3). A molecular weight of 440,000 for the native enzyme was obtained from sedimentation equilibrium by using a partial specific volume of 0.78 (2), which was experimentally measured by the D_2O method of Edelstein and Schachman (4). The high value of $\bar{v}$ might

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[6] Abbreviations used: BtChE, butyrylcholinesterase; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; PAS, periodic acid-Schiff reagent.

imply that BtChE from horse serum is a lipoprotein (5). Other reports suggested that BtChE contained sialic acid (6, 7) which, along with other carbohydrate residues, might cause the protein to behave anomalously both on gel filtration and in SDS-gel electrophoresis (8, 9). Recently Main *et al.* (10) reported a new value for $\bar{v}$ = 0.688. In view of the discrepancies between physical parameters of this protein (2, 3, 10) and because of problems which the carbohydrate content might cause, a major redetermination of several properties was undertaken. This paper reports the results for BtChE of amino acid analyses, N-terminal analyses, and determinations of carbohydrate composition. New data on the extinction coefficient, the partial specific volume, and molecular weights from sedimentation equilibrium measurements lead to a correction of earlier results (2) and resolution of the disagreement of results from separate laboratories, and they provide a more complete description of the chemical composition of BtChE.

EXPERIMENTAL PROCEDURES

Materials

Butyrylthiocholine iodide, β-naphthylacetate, and tetrazotized *O*-dianisidine (diazo blue-B) were obtained from Nutritional Biochemicals Corp. Triethylamine was purchased from Aldrich Chemical Co. Guanidine hydrochloride (ultra pure) and Coomassie brilliant blue were obtained from the Schwarz/Mann Division of Becton, Dickinson and Co. β-Mercaptoethanol was from E. H. Sargent and Co. Acrylamide, *N*-*N'*-methylene-bisacrylamide, *N*,*N*,*N'*,*N'*-tetramethyl ethylenediamine, and riboflavin were purchased from Eastman Kodak Co. Dansyl chloride and 5,5'-dithiobis(2-nitrobenzoic acid) were obtained from Sigma Chemical Co. Proteins used as molecular weight markers were from several commercial supply houses in the highest available purity. All chemicals were used without further purification.

Methods

Enzyme preparation and assay. BtChE was purified to homogeneity and the activity measured by methods previously described (1).

Amino acid analysis. Samples of purified BtChE in 6 N HCl were flushed with nitrogen, sealed in tubes under vacuum, and hydrolyzed for 24, 48, and 72 h at 110°C. The hydrolyzed samples were analyzed on a Beckman 119C automatic amino acid analyzer with the addition of norleucine as internal standard. Comparable measurements were made on a Beckman 120 analyzer at Brandeis University. Half-cystine was determined as cysteic acid after performic acid oxidation (11). Tryptophan content was determined spectrophotometrically (12). The number of free sulfhydryl groups was determined by titrating BtChE samples in the presence of 6 M Gdn·HCl with 5,5'-dithiobis(2-nitrobenzoic acid) (13). N-terminal analysis was carried out by the dansyl chloride method (14).

Carbohydrate composition. Analysis of carbohydrate was performed by gas–liquid chromatography as described by Clamp *et al.* (15), with the following modifications: (a) Erythritol and *meso*-inositol were employed as internal standards; (b) methanolysis was conducted with 1 N methanolic hydrochloric acid at 90°C for 4 h; and (c) separations were performed on a 12 ft × 1/8-in. stainless-steel column, containing Gas-Chrom Q (60/80 mesh) as the stationary support and 3% OV-17 (phenyl methyl silicone; 50% phenyl) as the liquid phase, with a temperature program of 120 to 250°C at 4°C/min. Chromatography was performed on a Hewlett–Packard Model 5700 gas chromatograph equipped with a Hewlett–Packard Model 3370B electronic integrator.

Lipid analysis. After disc polyacrylamide-gel electrophoresis, the gels were stained for lipid either with oil red O (16) or Sudan black B (17).

Protein determination. Routine protein concentrations were determined from measurements of uv absorption with the extinction coefficient reported in Results. Absorbances of proteins in 0.01 M potassium phosphate, pH 7.0, were measured directly or on gravimetrically diluted samples in a Cary 14 spectrophotometer at 280 nm. Corrections for light scattering contributions to absorbance were made for concentrated solutions ($A_{280\,nm} > 0.5$) with the procedures described by Leach and Scheraga (18) and Winder and Gent (19).

The extinction coefficient was obtained from absorbance measurements and from protein concentrations, determined both by dry weight and by differential refractometric methods. Dry weight measurements were carried out as described by Hunter (20) with the following modifications. The protein solutions were dialyzed against 0.01 M potassium phosphate buffer for 24 h and filtered through a Millipore filter with a 0.45-μm pore size. Aliquots containing approximately 1 mg of protein or appropriate amounts of buffer were delivered into preweighed, 10-ml volumetric flasks and dried *in vacuo* at 110°C for 36 h. Since the samples slowly gained weight after removal from the oven, the following procedure was used. The flasks were cooled in a desiccator over Drierite for 30 min before weighing

as a function of time in a Mettler M5 micro-balance with Drierite in the chamber. The dry weight was then obtained by extrapolating the measurements back to the time of removal from the oven. The drying process was repeated until three, constant, zero-time weights were obtained.

For the differential refractometric measurements, a Spinco Model E analytical ultracentrifuge was used as described by Babul and Stellwagen (21). Triplicate determinations with sucrose solutions of known concentrations gave a calibration constant of 3.12 ± 0.03 (standard deviation) fringes (mg of sucrose)$^{-1}$ ml^{-1}. A value of 4.05 ± 0.04 fringes (mg of protein)$^{-1}$ ml^{-1} was then obtained from the preceding calibration by using the refractive index increments, 0.144 ml/g for sucrose (22) and 0.187 ml/g for proteins (23), at 546 nm.

Partial specific volume. The partial specific volume, $\bar{v}$, was obtained from precise measurements of the densities of protein solutions and the buffer against which they had been dialyzed. The procedures described by Lee and Timasheff (24) were used to measure densities with a DMA 02 C Precision density meter (Anton Parr, Graz, Austria) at 25.00 ± 0.02°C. Apparent partial specific volumes, ϕ, were calculated from the following equation (24, 25):

$$\phi = (1/\rho_0)\left[1 - \frac{(\rho - \rho_0)}{c}\right],$$

where ρ_0 is the density of solvent, ρ is the density of protein solution, and c is protein concentration in grams per milliliter, determined as described above from uv absorbance measurements.

Polyacrylamide-gel electrophoresis. Disc polyacrylamide-gel electrophoresis was performed at pH 8.9 according to the method of Davis (26). The SDS analytical gel electrophoresis was carried out as described by Weber and Osborn (27). The gels were stained with Coomassie blue for proteins (26), with PAS for carbohydrate (28), and with oil red O (16) or Sudan black B (17) for lipid.

Sedimentation equilibrium. Molecular weight determinations were carried out by the high speed equilibrium method of Yphantis (29). Because of the discrepancies in reported molecular weights for BtChE, the best possible accuracy was required in the molecular weight measurements. The interference method was chosen instead of the uv scanner system used by Lee and Harpst (2), since the former gives greater accuracy in concentration measurement and is not subject to the experimental problems associated with the scanner (30, 31). The sedimentation equilibrium experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with an RTIC temperature control unit. A 12-mm path length, double sector, aluminum-filled Epon centerpiece with sapphire windows was used for all runs. Alignment of the Rayleigh interference optical system in the Model E was carried out as described in the Beckman–Spinco Manual. The interference patterns were analyzed on a Nikon 6 C Shadowgraph. The weight average molecular weights ($\bar{M}_w$) at each radial position were calculated by using a computer program developed by Hsu and Neet (32).

RESULTS

Electrophoresis

The purity of the BtChE preparations used in this study was routinely examined by disc polyacrylamide-gel electrophoresis. A single component was observed in gels stained either for protein with Coomassie blue or for carbohydrate with the PAS reagent. Splitting the gel lengthwise and staining one half for protein and the other for carbohydrate showed that the PAS band corresponds to the Coomassie blue band. The PAS bands also matched the Coomassie blue bands of dissociated subunits after SDS-gel electrophoresis. These data indicate that BtChE is a glycoprotein and that its subunits contain carbohydrate.

After disc gel electrophoresis, oil red O and Sudan black B were used to analyze qualitatively for lipid in BtChE. Neither stain indicated that lipid was present, even after the gels were loaded with 100 μg of BtChE. These data indicate that BtChE is not a lipoprotein. However, it is possible that a small amount (less than 5%) of lipid is associated with BtChE but is not detected by the method used (33).

Amino Acid Analysis

The amino acid composition of BtChE from horse serum is summarized in Table I. The results are averages from 24, 48, and 72 h hydrolyses in 6 N HCl on each of two different preparations. Only the values for threonine and serine required extrapolation to zero time. After a 24-h hydrolysis in 6 N HCl there was 3 and 9% destruction of threonine and serine, respectively. Six moles of cysteic acid per BtChE subunit were found. However, no sulfhydryl group was detected when BtChE in 6 M Gdn · HCl was titrated with 5,5′-dithiobis(2-nitrobenzoic acid). Therefore, all six cysteic acids appear to be

TABLE I

CHEMICAL COMPOSITION OF BtChE FROM HORSE SERUM

Residue	Number of residues (moles)/mole of subunit ($\bar{M}_w$ = 80,000)	Number of residues/1000 residues
Lysine	34 ± 1	52 ± 2
Histidine	5 ± 1	8 ± 1
Arginine	30 ± 1	46 ± 2
Aspartic acid	63 ± 1	97 ± 2
Threonine	32 ± 1	49 ± 1
Serine	35 ± 3	54 ± 5
Glutamic acid	64 ± 5	99 ± 8
Proline	36 ± 1	56 ± 2
Glycine	42 ± 1	65 ± 1
Alanine	32 ± 1	49 ± 1
Valine	31 ± 1	48 ± 2
Methionine	10 ± 1	15 ± 2
Isoleucine	21 ± 1	32 ± 1
Leucine	52 ± 3	80 ± 5
Tyrosine	20 ± 2	31 ± 3
Phenylalanine	36 ± 2	56 ± 4
Half-cystine	6 ± 1	9 ± 1
Tryptophan	15 ± 1	23 ± 2
Mannose	32 ± 4	49 ± 6
Galactose	17 ± 3	26 ± 4
N-acetylglucosamine	24 ± 5	37 ± 8
Sialic acid[a]	11 ± 2	17 ± 3

[a] Assumed to be *N*-acetylated; see text.

derived from disulfide linkages in BtChE. This conclusion supports an earlier inference, derived from viscosity measurements on BtChE in 6 M Gdn·HCl, that the protein contains disulfide linkages (2).

Carbohydrate Composition

Analysis of individual sugar residues was performed by gas–liquid chromatography as described in Methods. Two different enzyme preparations gave the same results within the reported uncertainties (Table I). Mannose, galactose, *N*-acetylglucosamine, and sialic acid were the only sugars found. The glucosamine was judged to be the *N*-acetyl derivative on the basis of the ratio of the methyl glycoside derivatives (15). Although the sialic acid group in the enzyme was not identified, it was measured as the *N*-acetyl derivative. These analyses indicate that BtChE is about 20% carbohydrate by weight.

N-Terminal Amino Acid

The dansyl chloride method for determining N-terminal amino acids was found to be satisfactory when applied to glycoproteins (34). Two-dimensional chromatography on silica gel slabs showed only one predominant spot, which was identified as dansyl-glutamate. Therefore, it appears that either all the subunits have the same N-terminal residue, glutamine or glutamate, or that other subunits do not have free N-terminal residues.

Protein Determination

Routine protein concentrations were determined from absorbances at 280 nm. Light scattering corrections (see Methods and Ref. (18, 19)) were made especially for concentrated solutions (1–5 mg/ml) used in measurements of densities and extinction coefficients. However, it was found that the corrections decreased the absorbances (280 nm) of BtChE solutions an average 4% and were essentially independent of the sample preparation in use and concentration. Furthermore, the absorbances of dilute BtChE solutions used for most other measurements were so low that scattering corrections were unreliable or negligible. For these reasons, and since appropriate results from the literature apparently do not include scattering corrections, the extinction coefficients reported in Table II do not include the corrections for light scattering.

The extinction coefficients, $E^{1\%}_{1\,cm}$ at 280 nm, determined from two methods for measuring concentrations, are summarized in Table II.[7] The values of $E^{1\%}_{1\,cm}$ at 280 nm for BtChE were the average results of multiple determinations with different preparations. Dry weight measurements gave an $E^{1\%}_{1\,cm}$ at 280 nm of 15.2 ± 0.3 for BtChE in 0.01 M potassium phosphate (pH 7.0). In control experiments a value of 6.6 ± 0.1 was obtained for bovine serum albumin, which agrees with the

[7] A protein concentration of 1% is 1 g/100 ml.

TABLE II

EXTINCTION COEFFICIENTS, $E^{1\%}_{1cm}$ AT 280 NM

Protein	Method used for concentration measurement		Literature values
	Dry weight[a]	Refractometry[a]	
BtChE	15.2 ± 0.3[b]	15.6 ± 0.2[c]	25[d]; 20[e]; 15[e]; 13.6[f]; 11.3[g]
Bovine serum albumin	6.6 ± 0.1[b]	6.9 ± 0.1[c]	6.6[h]
α-Chymotrypsin	–	20.5 ± 0.4[c]	20.0[i]

[a] See methods section.
[b] Four determinations (standard deviations indicated).
[c] Two determinations (average deviations indicated).
[d] Jansz and Cohen (35).
[e] Main *et al.* (3).
[f] Main *et al.* (10).
[g] Lee and Harpst (2).
[h] Cohn *et al.* (36).
[i] Dixon and Neurath (37).

reported value of 6.6 (36). Since the dry weight measurement is the most direct and independent method to determine concentrations in solution, the value of 15.2 ± 0.3 was selected as the extinction coefficient at 280 nm for BtChE at low concentrations. For concentrated solutions of BtChE which required light scattering corrections, the dry weight extinction coefficient, 14.6 ± 0.3, was used.

Extinction coefficients were also determined from protein concentrations obtained by differential refractometry (21) with the instrument calibration constant of 4.05 fringes (mg of protein)$^{-1}$ ml^{-1}, described in Methods. The extinction coefficient of BtChE from refractometry was 15.6 ± 0.2, which is in good agreement with the value obtained from dry weights (Table II). In parallel experiments, values for $E^{1\%}_{1cm}$ at 280 nm of 6.9 ± 0.1 and 20.5 ± 0.4 were found for bovine serum albumin and α-chymotrypsin, respectively. These values (without scattering corrections) are also in good agreement with results in the literature (Table II).

Partial Specific Volume

The partial specific volume of BtChE was determined in two different ways. Apparent partial specific volumes of BtChE at concentrations between 2 and 5 mg/ml in 0.01 M potassium phosphate, pH 7.0, were obtained directly from density and concentration measurements as described in Methods. Since there was no concentration dependence of ϕ within the precision of measurements, $\bar{v}$ was taken as the average of the values of ϕ, 0.723 ± 0.003 ml/g (average deviation). In addition a partial specific volume of 0.710 ml/g was calculated from the amino acid and carbohydrate compositions (38, 39) reported in Table I. In view of the high precision in the measurements of density and concentration and the inherent uncertainty in calculation of partial specific volume from chemical compositions, the value of 0.723 ± 0.003 ml/g was used in all subsequent calculations of appropriate physical parameters.

Molecular Weight

The molecular weight of native BtChE in 0.01 M potassium phosphate, 0.1 N NaCl, pH 7.0, was determined in high speed sedimentation equilibrium experiments. Plots of log f (fringe displacement) vs r^2 appeared to be linear throughout the liquid column. However, plotting these data as point weight average molecular weight vs protein concentration (Fig. 1) showed that BtChE exhibited a small, negative virial coefficient in this solvent. Figure 1 also illustrated that the native enzyme did not dissociate into subunits over the concentration range which was examined. A weight average molecular

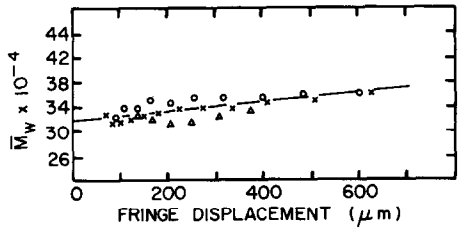

FIG. 1. Point weight average molecular weight vs concentration (given as fringe displacement in micrometers) from sedimentation equilibrium data in 0.1 N NaCl-0.01 M potassium phosphate, pH 7.0, at 12,000 rpm and 5°C. The initial protein concentrations were 0.12 mg/ml (△—△) and 0.6 mg/ml (×—× and ○—○). The line was drawn by least squares with equal weights for all points.

weight of 317,000 ± 12,000 (standard deviation) was obtained from the least-squares extrapolation to zero protein concentration (Fig. 1).

Subunit Molecular Weight of BtChE

High pH. Sedimentation equilibrium was also used to measure the subunit molecular weight of BtChE in 0.011 M triethylamine-sodium hydroxide at pH 11.8. Plots of log f vs r^2 were linear. It was assumed that the partial specific volume of BtChE did not change at high pH, so the value of 0.723 was used. One reliable run at 23°C gave a weight average molecular weight of 81,000 ± 1000 (standard deviation), obtained from a least-squares extrapolation to zero concentration (Fig. 2).

6 M Gdn·HCl. High speed sedimentation equilibrium was also performed at 23°C in 6 M Gdn·HCl, 0.1 M β-mercaptoethanol. Plots of log f vs r^2 were linear. It has been shown (40-42) that a decrease of 0.02 ml/g in the partial specific volume of native proteins may be expected when they are denatured in high concentrations of Gdn·HCl. On this basis the value of 0.703 was assumed to be the partial specific volume (ϕ') of the BtChE in 6 M Gdn·HCl. From this value of ϕ', the weight average molecular weight was calculated to be 79,000 ± 4000 (standard deviation for three separate determinations) from the zero concentration extrapolation of plots similar to the one shown (Fig. 2).

Lee and Timasheff (24) have shown that the partial specific volumes of different native proteins decrease from 0 to 0.03 ml/g in 6 M Gdn·HCl. Because of this variation in ϕ', it is desirable to have an estimate of the parameter for BtChE. A recently published method for calculating ϕ' in 6 M Gdn·HCl (43) gives $\phi' = 0.707$ for BtChE from the data in Table I and from the assumption that each residue of carbohydrate binds one molecule of Gdn·HCl. This value of ϕ' agrees well with the assumed value (0.703) in the preceding paragraph and yields a weight average molecular weight of 81,000 from the sedimentation equilibrium results for BtChE in 6 M Gdn·HCl.

0.1% SDS. The subunit molecular weight of BtChE was estimated by analytical SDS-polyacrylamide-gel electrophoresis (Figs. 3a and b). In both 5 and 10% gels, two bands were observed (stained either with Coomassie blue or PAS). The major, fast-moving band consisted of 70–75% and the minor, slow-migrating band had 25–30% of total protein, as estimated from relative Coomassie blue dye intensities. On the basis of the results from 5% gels (Fig. 3a), molecular weights of 90,000 and 162,000, respectively, were estimated for the major and minor bands. With 10% gels (Fig. 3b), corresponding values of 87,000 and 173,000 were obtained. From the ratio of the molecular weights and the relative dye intensities of the bands, it appeared that the slow-migrating, minor protein band was a dimeric form of the subunits, which did not dissociate under the conditions used.

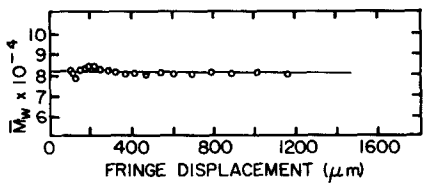

FIG. 2. Point weight average molecular weight vs concentration (given as fringe displacement in micrometers) from sedimentation equilibrium data for BtChE in 0.011 M triethylamine-sodium hydroxide, pH 11.8, at 20,000 rpm, 23°C. Initial BtChE concentration was 0.6 mg/ml. The line was drawn by least squares with equal weights for all points.

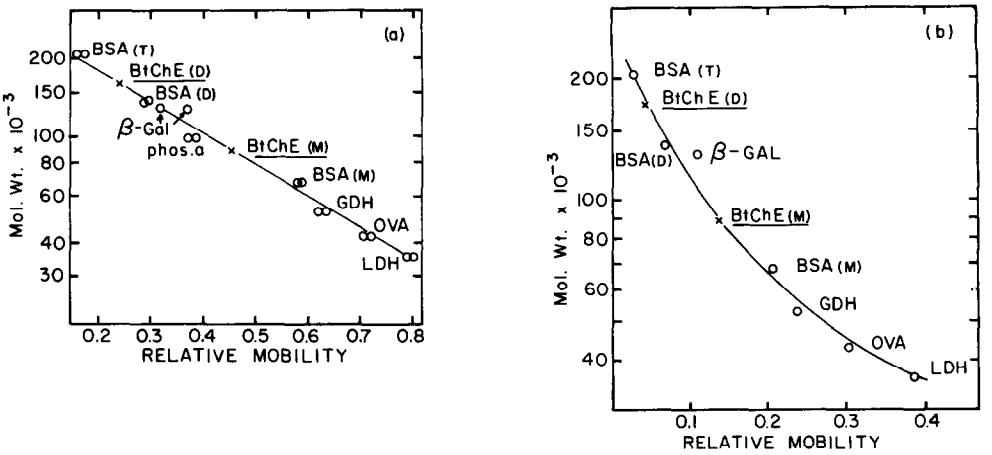

FIG. 3. Estimation of the subunit molecular weight of BtChE by 0.1% SDS analytical gel electrophoresis. Relative mobility is the ratio of migrating distance of protein to that of bromophenol blue. Standard proteins (○) and molecular weights were: BSA (T), bovine serum albumin, trimer, molecular weight 204,000; BSA (D), bovine serum albumin, dimer, 136,000; β-Gal, β-galactosidase, 130,000; phos. a, phosphorylase a, 100,000; BSA (M), bovine serum albumin, monomer, 68,000; GDH, glutamine dehydrogenase, 53,000; OVA, ovalbumin, 43,000; and LDH, lactate dehydrogenase, 36,000. Monomeric (M) and dimeric (D) forms of BtChE are indicated by (×). (a) 5% gels; the line was drawn by least squares with equal weights for all points. (b) 10% gels; the line was drawn by a nonlinear regression fit to the equation, $y = a_0 + a_1x + a_2x^2$.

DISCUSSION

Results from this investigation substantiate the relatively high carbohydrate content of BtChE reported by Main *et al.* (10). Earlier work had indicated that serum BtChE was a glycoprotein (6, 44). Heilbronn (7) reported that a partially purified preparation of BtChE from horse serum contained 3.24% sialic acid. This is equivalent to approximately nine sialic acid residues per subunit, which is in good agreement with the number, 11 ± 2, from Table I. Results from gel electrophoresis and from carbohydrate analysis (Table I) show that horse serum BtChE is a glycoprotein. The overall carbohydrate content (20%) is in fair agreement with the value of 17.3% determined recently by Main *et al.* (10), although there are relatively large differences in the amounts of individual sugars, possibly due to the analytical methods used. With respect to the distribution of different sugars, horse serum BtChE is similar to other typical serum glycoproteins, such as fetuin (45), α_1-acid glycoprotein (46), and transferrin (47).

The amino acid composition was determined in order to estimate $\bar{v}$ from chemical composition and to facilitate comparison with other work. The results in the last column of Table I are comparable to, but ten times larger than, the values of mole percent reported by Main *et al.* (10), except for the lower values of histidine and half-cystine and higher values of proline and phenylalanine. The composition of BtChE from porcine parotid glands has been reported by Tucci and Seifter (48) but no carbohydrate analysis was included. Even when the carbohydrate content is neglected, significant differences between the horse and porcine enzymes are found in several residues, particularly histidine, glycine, alanine, valine, methionine, isoleucine, and tyrosine. Whether this species difference is real or reflects any functional variations is not yet known.

A wide range of values for the extinction coefficient of BtChE from horse serum has been reported and is summarized in Table II. Main *et al.* (3) noted that after dialysis, freeze-drying and re-solution in phosphate buffer, the extinction coeffi-

cient of BtChE decreased from 20 to about 15. In order to avoid the possible complications due to freeze-drying and re-solution, neither the commercially available, crude material nor the purified BtChE preparations used in the present investigation have been freeze-dried.

As reported in Table II, the values of $E^{1\%}_{1\,cm}$ at 280 nm obtained from dry weight and differential refractometric measurements are 15.2 ± 0.3 and 15.6 ± 0.2, respectively. The accuracy and validity of these values are strengthened for the following reasons: (a) Values from dry weight measurements agree well with those from differential refractometry, and (b) the results of parallel experiments with bovine serum albumin and α-chymotrypsin standards are in good agreement with values in the literature (Table II). On the basis of these considerations and since the dry weight measurement is the most direct and reliable method for determining extinction coefficients, $E^{1\%}_{1\,cm}$ at 280 nm = 15.2 was chosen as the best value for future use. This result is moderately close to the value $E^{1\%}_{1\,cm}$ = 13.6 reported recently by Main *et al.* (10). The earlier result of 11.3 determined by Lee and Harpst (2) is considered to be incorrect.

The high carbohydrate content of BtChE raises a question about the validity of the results from differential refractometry. In Methods, calibration of the ultracentrifuge was based on the refractive index increment for proteins (23). The 20% carbohydrate content of BtChE could reduce *dn/dc* by a maximum of 5% if the sugar residues had the same *dn/dc* as sucrose (22). Such a change in *dn/dc* would reduce $E^{1\%}_{1\,cm}$ from refractometry to 14.5 ± 0.2, which is lower than the dry weight value but still in reasonable agreement with it. The data from dry weight and refractometric measurements provide a determination of *dn/dc* for BtChE, as shown in Table III. The value obtained, 0.183 ml/g, is lower than that for proteins (23) but is not as low as expected from the preceding estimate.

A partial specific volume of 0.78 ml/g was reported (2) on the basis of measurements by the D_2O method of Edelstein and Schachman (4). In the present investigation, a $\bar{v}$ value of 0.723 has been obtained from precise density measurements and 0.710 calculated from the amino acid and carbohydrate composition. The latter two values agree within experimental error with each other and with the value 0.688 ± 0.04 determined by Main *et al.* (10), but they are much lower than the result from D_2O. The D_2O method has been used to measure $\bar{v}$ for a large number of proteins (31). However, the high $\bar{v}$ obtained by Lee and Harpst (2), as well as other relatively high values of $\bar{v}$ obtained for glycoproteins with the same technique (49–51), may indicate that the D_2O method is not reliable when applied to this class of proteins. The high $\bar{v}$ does not seem to result from the choice of k for polypeptides (2, 4, 31), since a correction for the carbohydrate content of BtChE would increase both k and $\bar{v}$ over the previous values (2). Because of the

TABLE III

PHYSICAL PROPERTIES OF NATIVE BtChE[a]

Sedimentation coefficient, $s^0_{20,w}$	11.5 ± 0.5 S[b]
Molecular weight, M_w	(31.7 ± 1.2) × 10^4 g/mol
Partial specific volume, $\bar{v}$	0.723 ± 0.003 ml/g
Intrinsic viscosity, [η] at 25°C	10 ± 1 ml/g[c]
Extinction coefficient, $E^{1\%}_{1\,cm}$ at 280 nm (without light scattering correction)	15.2 ± 0.3
Refractive index increment at 546 nm, *dn/dc*	(1.83 ± 0.03) × 10^{-4} liter/g[d]

[a] All parameters were obtained in 0.01 M potassium phosphate at pH 7.0, except $\bar{M}_w$, which was measured in the same phosphate buffer with 0.1 N NaCl at pH 7.0.

[b] Taken from Lee and Harpst (2).

[c] Revised from the original measurements reported by Lee and Harpst (2).

[d] The concentration of BtChE solution was determined spectrophotometrically by using an $E^{1\%}_{1\,cm}$ of 15.2. A value of 3.97 fringes (mg of BtChE)$^{-1}$ml^{-1} was obtained from measurements of fringe displacement of BtChE solutions of known concentrations. Since $(dn/dc)_{BtChE}$:$(dn/dc)_{sucrose}$ = (f mg^{-1} ml^{-1})$_{BtChE}$:(f mg^{-1} ml^{-1})$_{sucrose}$, the value of $(dn/dc)_{BtChE}$ was calculated with values of $(dn/dc)_{sucrose}$ = 1.44 × 10^{-4} liter/g, (f mg^{-1} ml^{-1})$_{BtChE}$ = 3.97, and (f mg^{-1} ml^{-1})$_{sucrose}$ = 3.12.

inherent accuracy of the dry weight and density measurements, possible complications with the D_2O method due to the carbohydrate content of BtChE, and uncertainties in results from the scanner (31), the value of 0.723 presently appears to be the most reliable partial specific volume for BtChE.

When a $\bar{v}$ of 0.723 is used, $\bar{M}_w$ of native BtChE is 317,000 ± 12,000 from the sedimentation equilibrium data in Fig. 1 and 350,000 ± 35,000, when the sedimentation equilibrium results reported by Lee and Harpst (2) are corrected. The deviations indicated for both values were calculated from observed variations in multiple measurements with no assumed error in $\bar{v}$. These results agree within experimental uncertainty, particularly when the 10% error in the earlier result (2) is considered. However, the new value of 317,000 is more reliable because of elimination of the difficulties associated with the use of the scanner, referred to in Methods, utilization of a more accurate concentration extrapolation, and the consequent improvement in reproducibility. The $\bar{M}_w$ of 317,000 is lower than 368,000 reported for BtChE from porcine parotid glands, for which an assumed $\bar{v}$ of 0.73 was used (48), but is in good agreement with the value of 315,000 determined for horse serum BtChE by gel filtration (3). Although the molecular weight of 315,000 might be questioned because of the anomalous behavior suspected for glycoproteins (8), the good agreement of $\bar{M}_w$ values from sedimentation equilibrium and from gel filtration implies that the shape of BtChE does not deviate much from that of globular proteins, which are generally used as standards for molecular weight estimation by gel filtration.

The earlier determination of $\bar{M}_w$ for native BtChE by light scattering (2) was corrected for concentration with the new extinction coefficient (Table III). A molecular weight nearly twice as high as the preceding value (317,000) from sedimentation equilibrium measurements was obtained. Reevaluation of the data and calculations uncovered no explanation for this discrepancy. Since the molecular weight values discussed in the preceding paragraph are so consistent, it must be concluded that the light scattering determinations were in error. Further investigation is necessary to clarify the reasons for this discrepancy.

Subunit molecular weights of 79,000 ± 4000 and 81,000 ± 1000 were determined by sedimentation equilibrium in 6 M Gdn·HCl and in 0.011 M triethylamine–sodium hydroxide, pH 11.8, respectively. Correction of earlier results (2) with values of $\bar{v}$ used in this investigation decreased the subunit molecular weights (52) to values comparable with those reported here. Because of the improved techniques used in the present investigation (see Methods) and better precision in the results, the new subunit molecular weights near 80,000 are judged to be the most reliable.

Results from SDS analytical gel electrophoresis in both 5 and 10% gels (Fig. 3) gave an average molecular weight of 88,500, significantly higher than that from sedimentation equilibrium. It is known that SDS gels may give erroneous molecular weights for glycoproteins (9). Leach and Fish (53) have reported that the estimations of molecular weights of glycoproteins by SDS-gel electrophoresis are too high; for example, the estimated molecular weight is 15–20% high for a glycoprotein containing 25% carbohydrate. If an approximate correction of the molecular weight for BtChE monomers from SDS-gel electrophoresis is made on this basis (53), the subunit molecular weight is about 76,000, in fair agreement with the values from sedimentation equilibrium.

A subunit molecular weight of 77,300, obtained from SDS-gel electrophoresis, was reported by Main *et al.* (3). This is significantly lower than the value 88,500 obtained in the present investigation. The discrepancy may be explained by the linear extrapolation of the standard curve obtained by Main *et al.* (3), who appear to have used a gel concentration of 8–10%, as indicated by the relative mobilities of the standard proteins. Results on 10% gels shown in Fig. 3b clearly curve upward in the molecular weight region which includes BtChE monomers and larger stand-

ards. A linear extrapolation of data from standard proteins with molecular weights lower than that of BtChE monomers would lead to an underestimate of values in the higher molecular weight range (27).

Since the molecular weights for native and dissociated BtChE are 317,000 and 80,000, respectively, it is concluded that BtChE consists of four subunits with similar molecular weights. In spite of the changes in $\bar{M}_w$ necessitated by the alterations in $E^1_{1\,cm}$ at 280 nm and $\bar{v}$ discussed in preceding paragraphs, this conclusion is unchanged from the earlier one reached by Lee and Harpst (2) and from a similar inference made by Main *et al.* (3) on horse serum BtChE. It also parallels the conclusion reached by Scott and Powers (54) on the subunit structure of human serum BtChE. Available evidence, including the finding of a single N-terminal glutamate, suggests that the subunits of horse serum BtChE are identical, but this conclusion is still tentative.

An estimation of the general shape of native BtChE was attempted from hydrodynamic parameters (Table III) which have been obtained in this work and revised from the earlier report by Lee and Harpst (2). The ratio of frictional coefficients (f/f_0) for native enzyme is 1.50 (55), which gives a maximum axial ratio of 9 for a prolate ellipsoid (56), if hydration is assumed to be zero. A β value of 2.49×10^6 was calculated (57) from appropriate parameters at 25°C. This result suggests that BtChE is a prolate ellipsoid with an axial ratio of 13 (56). These interpretations of hydrodynamic results should be considered as tentative, due to the expected branched-chain characteristics of glycoproteins (58).

The present work demonstrates that horse serum BtChE is a glycoprotein consisting of 80% protein and 20% carbohydrate. The native BtChE has a $\bar{M}_w$ of 317,000 and is a tetrameric macromolecule, probably with four identical subunits.

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