A Partial Characterization of Acetylcholinesterase*

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The enzyme acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) has an important role in nerve functions. The enzyme was prepared from the electric tissue of the *Electrophorus electricus*, using column-chromatographic procedures. This preparation, the most active yet described, is nearly chromatographically pure and has a specific activity of 660 mmoles acetylcholine hydrolyzed/hour per mg of protein. The molecular weight was determined to be approximately 230,000, based on a sedimentation coefficient of 10.8×10^{-13} and a diffusion coefficient of 4.3×10^{-7} cm²/sec. On the basis of equivalent weight determinations the sedimenting species of the enzyme contains four active sites. By the methods used, sedimentation, diffusion, and gel filtration, no evidence was found for the existence of a substantially higher- or lower-molecular-weight species of the enzyme. In gel-filtration studies with an agar column the enzyme moved with catalase, suggesting a molecular weight of about 250,000. The friction ratio was calculated to be 1.25, which is indicative of a globular protein.

The enzyme acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7.) is difficult to purify and consequently the molecular properties of the enzyme have not been extensively studied. The most highly purified preparations of the enzyme have been obtained from the electric organ of the "electric eel," *Electrophorus electricus*. Procedures for the purification of the enzyme have been described (Rothenberg and Nachmansohn, 1947; Lawler, 1959) by which a preparation having a specific activity of 400 (mmoles of acetylcholine hydrolyzed/hour per mg protein) was obtained. This preparation is characterized by a molecular weight of 13–31 million (Lawler, 1963).

A chromatographic procedure for the purification of the enzyme (Hargreaves *et al.*, 1963) has been utilized to obtain a preparation having a specific activity of 40; this preparation on ultracentrifugation gave $s_{20,w}$ values of 4, 6.05, and 14 S, which might correspond to molecular weights of approximately 70,000, 100,000, and 300,000.

The most active preparation of the enzyme yet described has a specific activity of 660, and is nearly chromatographically homogeneous (Kremzner and Wilson, 1963). Some of the properties of this acetylcholinesterase preparation are described here.

Experimental Procedure

The enzyme acetylcholinesterase was purified by a chromatographic procedure previously described in detail and outlined in Table I. The enzyme tissue source was the main electric organ of *Electrophorus electricus*. Protein concentrations were determined from optical absorption measurements at 215 and 225 mμ (Murphy and Kies, 1960). The quantity of protein, in mg/ml, was obtained by multiplying the difference in optical density at 215 and 225 mμ by the factor 0.157. It previously has been shown that protein values estimated by this technique with this factor are in good agreement with values obtained by the ultramicro-Kjeldahl method for tne enzyme solutions obtained during this procedure.

TABLE I
PURIFICATION PROCEDURE FOR ACETYLCHOLINESTERASE[a]

Step	Process
(1)[b]	Electric tissue extracted with toluene
(2)[b]	Homogenized in 5% ammonium sulfate; centrifuged 30,000 × *g*
(3)	Chromatographed on benzyl-DEAE-cellulose
(4)	Enzyme concentrated by absorption on benzyl-DEAE-cellulose
(5)	Gel filtration, Sephadex G-200
(6)	Chromatographed on phosphorylated cellulose
(7)	Chromatographed on DEAE-cellulose
(8)	Chromatographed for a second time on DEAE-cellulose (specific activity of peak = 660)

[a] Kremzner and Wilson (1963). [b] Rothenberg and Nachmansohn (1947).

Enzyme activities were determined by the colorimetric method (Hestrin, 1949) and the continuous-automatic-titration method (Wilson and Cabib, 1954). The enzyme was concentrated, when necessary, by first diluting the enzyme solution to about 0.09 M salt with 0.02 M sodium phosphate buffer, *p*H 7.0, and applying the resultant solution to a minimum-volume column of DEAE-cellulose. By this procedure 5 or 10 mg of protein present in 150 ml of solution can be completely absorbed on a column having a volume of 1 ml or less, at a flow rate of approximately 50 ml/hr. The enzyme was then eluted with 1.0 M sodium chloride in 0.02 M sodium phosphate buffer, *p*H 7.0.

Changes of enzyme solvent were effected by using a minimum volume Sephadex G-25 column equilibrated with the desired solvent. The Sephadex procedure was adopted, in preference to dialysis, because of its rapidity.

The sedimentation velocity experiments were performed in a Spinco Model E ultracentrifuge with the schlieren optical system, the An-D rotor and the 2° single-sector cell with a 12-mm centerpiece (0.41-ml capacity). All determinations were made in 0.10 M sodium chloride, 0.03 M sodium phosphate buffer, *p*H 7.0, at a temperature of 20°. The sedimentation coefficients were calculated from measurements made from the photographic plates with the Gaertner micrometer comparator.

Diffusion measurements were made under conditions

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of free-solution diffusion in a multilayered ground-glass diffusion cell[1] (Moore *et al.*, 1959). All measurements with acetylcholinesterase were made at 4 ± 0.5°, with diffusion times of 50–65 hours. In very dilute solution the enzyme activity is not stable; to stabilize activity, crystalline bovine serum albumin was used. Sucrose was used to aid in the establishment of the initial boundary and to reduce convection. The enzyme preparation in 1% sucrose, 0.02% albumin, 0.10 M sodium chloride, and 0.03 M sodium phosphate buffer, *p*H 7.0, was placed in the bottom cell. The upper layers of the cell were filled with a solution of 0.02% albumin, 0.10 M sodium chloride, and 0.03 M sodium phosphate, *p*H 7.0. The rate of enzyme diffusion was determined by assaying enzyme activity in the individual cell compartments, after cleaving the cell. The data were analyzed and the diffusion constant was calculated by using tables computed by Trautman and Breese (1960). To check the method in our hands we determined the diffusion coefficient of crystalline human serum albumin. In this case no protein was added to the upper layers and protein was measured by the Lowry modification of the Folin method (Lowry *et al.*, 1951).

The normality of the enzyme solutions was determined by "titration" of the enzyme with the potent acetylcholinesterase inhibitor *N,N*-dimethyl-*S*-diethoxyphosphorylthioethanolamine (bioxalate salt)[2] (Tammelin, 1957). This inhibitor was used in preference to the quaternary compound diethylphosphorylthiocholine iodide because it can be more easily obtained in a pure form.

The pseudo-first-order rate constant for the inhibition of the enzyme was found to be 1.2×10^6 liters/mole min^{-1} in this work. In a typical "titration" an enzyme solution, which was about 7×10^{-7} normal with respect to active sites, was incubated with several concentrations of inhibitor in the concentration range 1.5–6 $\times 10^{-7}$ M. The incubation time was taken sufficiently long so that the reaction approached within 1% of completion as judged by calculation from the integrated second-order rate expression. Inhibitions greater than 80% were not attempted because the times required become excessively long (greater than 25 first-order half-times). Completeness of the reaction was checked by assaying again at a considerably longer time. The per cent inhibition was proportional to the concentration of inhibitor; "extrapolation" to 100% inhibition yields the concentration of enzyme sites or the normality of the enzyme solution. The activity of the enzyme solution divided by the normality of the solution gives the activity per mole of sites.

Gel-filtration studies, used to estimate the molecular weight of relatively impure enzyme preparations, were made with Sephadex G-200 and G-100, and gels of 5 and 7% agar (Andrews, 1962). The agar columns were standardized using proteins (also RNA) of known molecular weight, RNA, molecular weight 10^6;[3] bovine fibrinogen, 350,000; catalase, 250,000; alcohol dehydrogenase, 150,000; bovine serum albumin, 70,000; egg albumin, 44,000; and noting their peak elution volumes. The determinations were made in the presence of 0.10 M sodium chloride and 0.05 M sodium phosphate buffer, *p*H 7.0.

[1] Precision Cells, Inc., New York City.

[2] Prepared by Dr. Sara Ginsburg, Dept. of Neurology, Columbia University College of Physicians and Surgeons.

[3] Molecular weight determined by sedimentation velocity. Gift from Dr. H. S. Rosenkranz, Department of Microbiology, Columbia University College of Physicians and Surgeons.

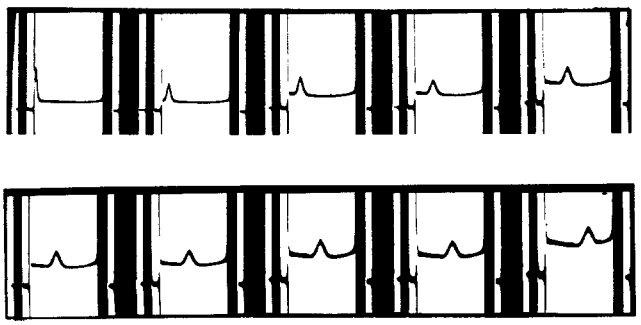

FIG. 1.—Sedimentation pattern of acetylcholinesterase with a specific activity of 660. The protein concentration was 1 mg/ml. Rotor speed was 36,950 rpm. Following the initial photograph, the next four frames were made at 16-minute intervals, followed by 8-minute intervals; the last frame was made after an 18-minute interval.

RESULTS

Sedimentation Studies.—The sedimentation pattern of the preparation of acetylcholinesterase with the highest activity, specific activity 660, showed the presence of a single symmetrical peak (Fig. 1). The $s_{20,w}$ value was found to be 10.8 S. Because of limitations in the quantity of specific activity–660 enzyme available it was not possible to determine the dependence of the sedimentation coefficient on protein concentration. However, the same sedimentation coefficient was obtained for concentrations 1.0 mg/ml and 0.70 mg/ml for enzyme with specific activity 660. Sedimentation-velocity measurements also were made with acetylcholinesterase preparations having specific activities of 320 and 460. Again photographs showed only a single symmetrical peak and the sedimentation coefficient was the same as found for the purest enzyme preparation. The sedimentation coefficients were the same with these preparations, in the concentration range 2.5–0.70 mg/ml. In two cases when an enzyme preparation having an original specific activity of 600 or greater lost considerable activity on storage, the $s_{20,w}$ value increased to 11.4 S.

Diffusion.—The diffusion studies were conducted with acetylcholinesterase of specific activity 100–660 using solutions containing from 0.1 to 0.05 mg protein/ml for the more active preparations and correspondingly more protein in the less active preparations. The diffusion coefficient was found to be 2.6×10^{-7} cm^2/sec at 4°, which calculates to 4.3×10^{-7} cm^2/sec at 20° on the basis that the diffusion coefficient is proportional to the ratio of temperature and viscosity and with the assumption that there is no change in conformation between 4 and 20°.

The diffusion coefficient of human serum albumin was found to be 4.1×10^{-7} at 6°, which by calculation gives rise to 6.3×10^{-7} cm/sec^2 for 20°. This value compares reasonably well with the published value of 6.1×10^{-7} cm/sec^2 (Oncley *et al.*, 1947).

Molecular Weight.—The molecular weight of acetylcholinesterase was calculated from Svedberg's equation, assuming a value of 0.73 for the partial specific volume, to be 230,000. This value is, of course, only approximate because we have assumed the value of the partial specific volume and have corrected the diffusion coefficient from 4°.

In order to determine if the preparations contained other molecular weight forms of acetylcholinesterase, column-gel-diffusion experiments were conducted. Lower-molecular-weight forms of the enzyme were sought using Sephadex G-200 and G-100; evidence for higher- or lower-molecular-weight species was sought

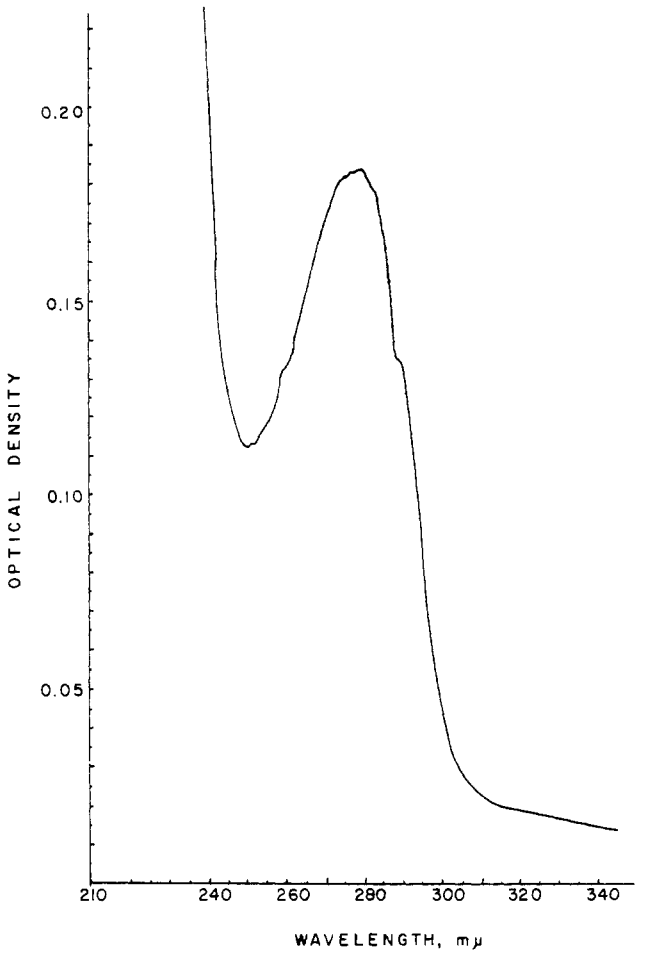

FIG. 2.—Absorption spectrum of acetylcholinesterase in 0.10 M sodium chloride and 0.03 M sodium phosphate buffer, *p*H 7.0. The protein concentration is 0.08 mg/ml; specific activity, 660. Manually, Beckman Model DU spectrophotometer; wavelength intervals were 0.5 mμ in critical regions and 1.0 mμ at other wavelengths.

by filtration on columns of 5 and 7% agar. The enzyme preparations examined were: (1) a freshly prepared tissue homogenate, centrifuged at 30,000 × *g*; (2) an enzyme solution of low specific activity 12, prepared from toluene-extracted tissue by ammonium sulfate fractionation (Lawler, 1959); (3) enzyme solutions prepared by the chromatographic procedure, at various stages of purification, specific activity 25, 60, 300, 400, 600. All the known proteins (and RNA) passed through the column in the order of decreasing molecular weight as reported by Andrews (1962). Within the limits of the assay error, approximately ±5%, no evidence was found for the existence of either a higher- or lower-molecular-weight form of the enzyme in any of the above preparations. All the acetylcholinesterase activity in every solution appeared in the effluent from the agar columns with catalase and distinctly before alcohol dehydrogenase. It was noted that the enzyme is retarded slightly on passage through a column of Sephadex G-200. From gel filtration the molecular weight appears to be roughly 250,000.

Activity per Site.—The activity per mole of active sites was found to be 6.1 × 10^5 μmoles/min for enzyme preparations having specific activities between 300 and 660. This value is somewhat lower than the value measured previously, 7.4 × 10^5, by a different method for a preparation obtained by ammonium sulfate fractionation (Wilson and Harrison, 1961). A crude preparation, specific activity 12 obtained by ammonium sulfate fractionation, was checked by the method used in this work and a higher activity per site, 7.0 × 10^5 μmoles/min, was obtained. It appears therefore, that the lower activity per site measured with the highly purified preparations obtained by our chromatographic method is real.

The equivalent weight (the weight per active site) can be obtained by dividing the activity per site by the specific activity (expressed in minutes) of a *pure* preparation. Assuming, for the purposes of this calculation, that our preparation with the highest specific activity 660 (11 expressed in minutes) is approximately pure, we obtain 54,000 as the equivalent weight. It is clear that the sedimenting unit contains more than one active site—the best value at this time is four. It appears then that the enzyme molecule may consist of four subunits.

Absorption Spectrum.—The ultraviolet-absorption spectrum of the enzyme, Figure 2, is characterized by an absorption maximum and minimum at 280 and 250 mμ, with distinct fine structure at 290, 278, and 258 mμ. The absorption at 280 mμ is 2.29/mg per ml (10 mm path); the ratio of the absorption at 280:250 mμ is 1.67.

DISCUSSION

Acetylcholinesterase has a lower molecular weight (230,000) than was previously thought. This value obtained by sedimentation-diffusion was confirmed by gel filtration within the approximate evaluation possible with this technique. Since gel filtration yields the same value for fresh tissue extracts and crude preparations, as well as the highly purified solutions obtained by chromatography, it would appear that the very high molecular weight obtained by other preparative methods is a consequence of the procedure.

It should be emphasized that the evaluation of the equivalent weight is not a primary measurement but depends upon the value taken as the specific activity of pure enzyme. The value calculated in this work (54,000) is based upon the assumption made for this purpose that the specific activity of pure enzyme is not much greater than 660. This is not unreasonable since preparations with this activity are nearly chromatographically homogeneous. Since a higher specific activity would yield a lower equivalent weight, it is quite certain that the molecular unit involved in sedimentation and diffusion (230,000) contains at least four active sites. The best estimate from the present data is four. There is no information, at this time, bearing on whether the enzyme might be dissociated into "four" subunits or whether such units would be independently active.

The inadequacy of "a single sedimenting peak" as evidence of purity is clearly indicated in this work; neither the $s_{20,w}$ value nor the appearance of the peak changed as the preparation was purified from specific activity 300 to 660. On the other hand the chromatographic distribution of protein changed. This is, of course, the basis of the purification procedure. In those cases where there was an extensive loss of activity on prolonged storage of very active (>600) preparations, most of the remaining activity could be recovered in the form of a solution with very high specific activity by repeating the last step of the procedure: chromatography on DEAE-cellulose.

The data can be used to calculate the ratio of friction coefficients f/f° where $f = kT/D$ and $f^\circ = 6\pi a\eta$, the coefficient for an unhydrated equivalent sphere with the radius a calculated from the molecular weight; η is the viscosity. This ratio comes out to be 1.25.

This number is approximate, but even allowing for error the value lies in the range typical of globular proteins.

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A Water-insoluble Polyanionic Derivative of Trypsin. I. Preparation and Properties*

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A number of water-insoluble polyanionic derivatives of trypsin (IMET) were prepared by coupling the enzyme to a copolymer of maleic anhydride and ethylene, cross-linking with hexamethylenediamine, and hydrolyzing the unreacted maleic anhydride residues. Different IMET preparations with varying bound protein-to-carrier ratios (from 1:20 to 3:1) were obtained. Their esteratic activities per unit weight of bound protein corresponded to 40–70% of that of crystalline trypsin. All of the IMET preparations studied were found to be considerably more stable than trypsin in the alkaline *p*H range (*p*H 7.0–10.7). The polyelectrolyte trypsin derivatives, IMET-2 and IMET-8, retained practically all their enzymatic activity on storage for 3–8 months under 0.1 M phosphate buffer of *p*H 7 at 4°. The IMET preparations could be lyophilized and kept in dry form, at room temperature, without marked loss in activity. IMET samples rich in carrier retained some of their enzymatic activity in 8 M urea. The digestion of the high-molecular-weight substrates casein, hemoglobin, lysozyme, and poly-L-lysine by the various IMET preparations has been investigated. Chymotrypsinogen could be activated to chymotrypsin by IMET-1 and IMET-6 as well as by a water-insoluble polytyrosyl trypsin derivative. Most of the esterase activity toward benzoyl-L-arginine ethyl ester of the various IMET preparations could be inhibited by pancreatic trypsin inhibitor at a 1:1 weight ratio of inhibitor to bound protein. Practically no inhibition of the esteratic activity by soybean-trypsin inhibitor occurred even at a 50:1 weight ratio of inhibitor to bound enzyme protein. The soybean-trypsin inhibitor was found, however, to be an effective inhibitor of the proteolytic activity of the IMET's as tested on casein. The *p*H-activity profiles of an IMET preparation rich in carrier (IMET-1) and of an IMET preparation rich in protein (IMET-6) at different ionic strengths were determined.

The preparation of a water-insoluble carboxymethylcellulose derivative of trypsin was reported by Mitz and Summaria (1961). The water-insoluble preparation obtained was found to be more stable than native trypsin. Epstein and Anfinsen (1962) used a preparation of trypsin bound to carboxymethyl-cellulose in their experiments on the reversible reduction of the disulfide bonds of this enzyme. Water-insoluble polytyrosyl derivatives of trypsin (IPTT)[1] were obtained in this laboratory (Bar-Eli and Katchalski, 1960, 1963) by coupling polytyrosyl trypsin (Glazer *et al.*, 1962) with a water-insoluble polydiazonium salt derived from a copolymer of *p*-amino-DL-phenylalanine and L-leucine. The water-insoluble polytyrosyl trypsin preparations showed an esteratic activity per unit weight of bound protein corresponding to 15–30% of that of crystalline trypsin. Lower enzymatic activities were recorded for water-insoluble trypsin derivatives obtained by coupling unmodified trypsin with the water-insoluble carrier. The water-insoluble enzyme preparations were considerably more stable in the alkaline *p*H range (*p*H 7–9) than either trypsin or polytyrosyl trypsin. A column possessing tryptic activity was prepared from water-insoluble polytyrosyl trypsin.

In this paper a description is given of the preparation and properties of a new type of water-insoluble trypsin derivative in which trypsin is covalently bound to a

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[1] The abbreviations used are: IPTT, water-insoluble polytyrosyl trypsin; IMET, water-insoluble copoly-(maleic acid–ethylene)-trypsin; BAEE, benzoyl-L-arginine ethyl ester; ATEE, acetyl-L-tyrosine ethyl ester.