Jefferson, L. S., Koehler, J. O., and Morgan, H. E. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 816.
Jefferson, L. S., and Korner, A. (1969), *Biochem. J. 111,* 703.
Manchester, K. L. (1961), *Biochem. J. 81,* 135.
Manchester, K. L. (1970a), *Biochim. Biophys. Acta 213,* 532.
Manchester, K. L. (1970b), *Hormones 1,* 342.
Manchester, K. L., and Harris, E. J. (1968), *Biochem. J. 108,* 177.
Manchester, K. L., and Wulwick, C. (1969), *Protein Polypeptide Horm., Proc. Int. Symp., 1968,* 828.
Manchester, K. L., and Young, F. G. (1961), *Vitam. Horm. (New York) 19,* 95.
Morgan, H. E., Jefferson, L. S., Wolpert, E. B., and Rannels, D. E. (1971), *J. Biol. Chem. 246,* 2163.
Morgan, H. E., Rannels, D. E., Wolpert, E. B., Giger, K. E., Robertson, J. W., and Jefferson, L. S. (1972), *in* Insulin Action. Fritz, I. B., Ed., New York, N. Y., Academic Press, p 437.
Mortimore, G. E. (1961), *Amer. J. Physiol. 200,* 1315.
Pestka, S., Goorha, R., Rosenfeld, H., Neurath, C., and Hintikka, H. (1972), *J. Biol. Chem. 247,* 4258.
Pilkis, S. J., and Korner, A. (1971), *Biochim. Biophys. Acta 247,* 597.
Turner, L. V., and Manchester, K. L. (1973a), *Biochim. Biophys. Acta 299,* 612.
Turner, L. V., and Manchester, K. L., (1937b), *Biochim. Biophys. Acta 320,* 352.
von der Decken, A., and Omstedt, P. T. (1970), *J. Nutr. 100,* 623.
Wannemacher, R. W., Cooper, W. K. C., and Muramatsu, K. (1970), *Proc. Soc. Exp. Biol. Med. 135,* 180.
Williamson, A. R. (1969), *Biochem. J. 111,* 515.
Wool, I. G., and Kurihara, K. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 2401.
Wool, I. G., Stirewalt, W. S., Kurihara, K., Low, R. B., Bailey, P., and Oyer, D. (1968), *Recent Progr. Horm. Res. 24,* 139.
Young, V. R., and Alexis, S. D. (1968), *J. Nutr. 96,* 255.
Zak, R., Etlinger, J., and Fischman, D. A. (1972), *Excerpta Med. Found., Int. Congr. Ser. No. 240,* 163.

Structure of 11S Acetylcholinesterase. Subunit Composition†

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ABSTRACT: The subunit compositions of several 11S acetylcholinesterase (EC 3.1.1.7) preparations from the electric eel were investigated. Data from polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate and gel exclusion chromatography in 6 M guanidine hydrochloride indicated that the active enzyme is a tetramer composed of subunits with molecular weights of 70,000. From analyses of ^{32}P-phosphorylation patterns and cyanogen bromide fragment compositions, the subunits were shown to contain one active site each and to be identical within the limits imposed by these techniques, except for a variability in manifesting two sites of cleavage, probably caused by proteolytic or glycolytic agents. One cleavage occurs at a site A and splits the intact subunit (I) into a major fragment (II) of 50,000 molecular weight and a minor fragment (III) of 20,000–22,000 molecular weight. A second cleavage at a site B generates a second minor fragment (IV), with a molecular weight of 18,000–20,000, from III. In the absence of disulfide reduction these cleavages did not appear to result in the release of measurable polypeptides from the subunit structures. In the native enzyme both cleaved and intact subunits appear to exist as subunit dimers with a covalent intersubunit linkage which involves disulfide bonding. Hence the subunits in the native tetramer are arranged as a dimer of dimers $((\alpha)_2)_2$, where α is either the cleaved or intact subunit containing the catalytic site.

Acetylcholinesterase (EC 3.1.1.7) from the electric eel *Electrophorus electricus* has been isolated in each of several molecular species. Species isolated from extracts of fresh electric organ tissue are characterized by sedimentation coefficients of 8 S, 14 S, and 18 S and appear in electron micrographs as clusters of respectively 4, 6–8, and 10 or more subunits attached to an elongated "tail" (Rieger *et al.*, 1973; Dudai *et al.*, 1973). All three of these species may be converted to an 11S form either by treatment with trypsin or other purified proteolytic enzymes, or by an apparent "autolysis" on storage of crude enzyme solutions (Massoulié and Rieger, 1969; Rieger *et al.*, 1972a,b). Electron micrographs of the 11S species made in parallel with the other forms show globular structures composed of four subunits without the "tail."

The acetylcholinesterase properties reported here refer to an 11S enzyme purified by affinity chromatography from extracts of toluene-stored electric eel tissue (Rosenberry *et al.*, 1972; Chen *et al.*, 1974). Several purified preparations free of detectable protein contaminants have been obtained, and the physical

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[1] Abbreviations used are SDS, sodium dodecyl sulfate; DFP, diisopropylphosphoryl fluoride; DIP-acetylcholinesterase, diisopropylphosphorylated acetylcholinesterase; NEM, *N*-ethylmaleimide; IAA, iodoacetamide; PAS, periodic acid–Schiff reagent; DTT, dithiothreitol.

properties of the enzyme in its native state in these preparations are quite reproducible. However, variations in the relative polypeptide composition among these preparations are observed after disulfide reduction and electrophoresis on gels containing SDS.[1] The pattern of these variations suggests that the observed multiple polypeptides arise from fragmentation of a single polypeptide chain (Chen *et al.*, 1974). The results presented here confirm this suggestion and introduce several observations involving intersubunit relationships in the native enzyme tetramer. In a subsequent report (T. L. Rosenberry, manuscript in preparation) the presence of an intersubunit disulfide linkage is investigated in detail.

Experimental Section

Materials

Acetylcholinesterase. The enzyme was purified by affinity chromatography of extracts from toluene-stored eel tissue and characterized as an 11S species as described previously (Rosenberry *et al.*, 1972; Chen *et al.*, 1974). The three preparations used in the studies reported here are preparations 3 and 4 described in Chen *et al.* (1974) and preparation B (Figure 3B, Chen *et al.*, 1974). Preparations 3 and 4 were stored as frozen concentrates (20 mg of protein/ml) in 20 mM sodium phosphate (pH 7), and preparation B was stored at 5° in the same buffer. According to ^{32}P-labeling patterns, 6% of the enzyme subunits in preparation 3 and 45% of the subunits in preparation 4 were in the form of the intact polypeptide I (see Results). An estimated 20% of preparation B was in this form.

Protein Molecular Weight Standards. The following highly purified proteins were used to calibrate both SDS gels and Sepharose columns. The assumed molecular weight is listed in parentheses after each protein: rabbit phosphorylase A (Sigma) (92,500, Seery *et al.*, 1970); equine transferrin (NBC) (76,600, Mann *et al.*, 1970); bovine serum albumen (Mann) (68,500, Castellano and Barker, 1968); bovine γ-globulins (Sigma) (51,600 and 23,500, Waxdal *et al.*, 1968); ovalbumen (Sigma) (44,000, Castellano and Barker, 1968); porcine pepsin (Worthington) (33,000, Williams and Rajagopalan, 1966); conconavalin A (Pharmacia) (25,700, Waxdal *et al.*, 1971); bovine hemoglobin (Sigma) (15,500, Weber and Osborn, 1969); equine cytochrome *c* (Sigma) (11,700, Weber and Osborn, 1969); [^{125}I]-α-bungarotoxin (Miami Serpentarium, see Chang, 1974) (8000, Lee, 1970); bovine insulin (Sigma) (2300 and 3400, Swank and Munkres, 1971).

Radioactive Labels. [^{32}P]DFP (40–60 Ci/mol; Amersham-Searle) was used directly without addition of unlabeled DFP. Labeling was carried out within 1 week of receipt of the [^{32}P]DFP unless otherwise indicated. We observed that exposure of acetylcholinesterase to one initially satisfactory shipment of [^{32}P]DFP which had been stored in our laboratory for 2 months resulted in a degradative effect upon the apparent polypeptide molecular weights. The stoichiometry of [^{32}P]DFP acylation was in general agreement with that of other active site titrants (Rosenberry *et al.*, 1972); however, a variability among [^{32}P]DFP stocks was observed which indicated that the accuracy of the labeled [^{32}P]DFP specific activity was ±25%.

[^{14}C]NEM (2.5 Ci/mol; Amersham-Searle) was also used directly without addition of unlabeled NEM; with this reagent the concentration of the shipment was checked by uv absorbance measurements (λ_{max} 305 nm (ϵ_{305} 620); Gregory, 1955).

Reagents. Electrophoresis grade acrylamide and bisacrylamide were from Bio-Rad; ultrapure urea was from Mann; carrier ampholytes were from LKB-Producten; modified Schiff reagent was from Fisher; Coomassie Brilliant Blue R-250 was from Colab; and pyronin Y was a gift of Dr. A. Deitch, Columbia University. Other chemicals were all of reagent grade unless otherwise indicated.

Sample preparations in 6 M guanidine hydrochloride were carried out with the use of ultrapure reagent (Schwarz/Mann) unless otherwise indicated. Large volumes of 6 M guanidine hydrochloride used in column chromatography involved practical grade reagent (Eastman) purified in the following manner. Guanidine hydrochloride (1 kg; Eastman practical) was dissolved in 3 l. of 95% ethanol. The solution was vacuum-filtered twice (Whatman 3 paper); 100 g of Norit was added, and the solution was stirred for 45 min at 40–50°. After an additional vacuum filtration as above, 2200 ml of solvent was removed on a rotary evaporator accompanied by crystallization of the guanidine hydrochloride. The solution was cooled at 4° for 15 min, and the crystals were removed by vacuum filtration (Whatman 54 paper) and dried overnight at 40°. The yield was 750 g. The absorbance at 280 nm of aqueous 6 M guanidine hydrochloride prepared from the crystals was <0.08. Impurities caused the absorbance to rise sharply below 275 nm, but these impurities could be dialyzed completely.

Methods

Protein Determination. The concentrations of solutions of native acetylcholinesterase were determined with the use of $E_{280\,nm}$ (1%) 18.0 (Rosenberry *et al.*, 1972). The OD_{280} of acetylcholinesterase in 6 M guanidine hydrochloride was 8% lower than that in dilute phosphate buffer, corresponding to an extinction coefficient $E_{280\,nm}$ (1%) 16.6.

Acylation with [^{32}P]DFP. To 1–25 mg of acetylcholinesterase (5–15 mg/ml) in 50–100 mM sodium phosphate buffer (pH 7.0) was added [^{32}P]DFP at a final concentration of 0.5 mM. The solution was incubated for 1–2 hr at room temperature, and the resulting [^{32}P]DIP-acetylcholinesterase was dialyzed against 10–20 mM sodium phosphate buffer (pH 7.0) overnight.

Alkylation of Disulfide-Reduced [^{32}P]DIP-acetylcholinesterase with [^{14}C]NEM. Reduction of 25 mg of [^{32}P]DIP-acetylcholinesterase (3.0 mg/ml, preparation 4 after 3 months of storage) was carried out in an initial excess of DTT (1 mM) at pH 9 for 2 hr at 50° in 50 mM Tris-Cl containing 6 M guanidine hydrochloride. The sample had been preincubated at 50° in the reduction solvent for 4 hr prior to the addition of DTT. The oxidation of DTT was followed spectrophotometrically; oxidized DTT has an absorbance band tail outside the protein absorbance (for oxidized DTT, λ_{max} 283 nm (ϵ_{283} 273); (ϵ_{315} 80); see Cleland, 1964). After adjustment of the pH to 6–7 with 1 N HCl, the sample was alkylated without dialysis by the addition of [^{14}C]NEM to a concentration of 0.6 mM, an amount threefold in excess of the total remaining free thiol concentration, for 2 hr at 25° (see T. L. Rosenberry, manuscript in preparation). The major portion of the ^{32}P,^{14}C-labeled sample was immediately introduced to column chromatography; a small remaining portion was dialyzed against 20 mM sodium phosphate (pH 7) containing 0.6 M guanidine hydrochloride (column grade) for 3 days at 25° for ^{14}C stoichiometry measurements. An evaluation of the [^{14}C]NEM-labeling stoichiometry obtained with this procedure showed that about 75% of the maximal enzyme alkylation had occurred (T. L. Rosenberry, manuscript in preparation).

Protein Alkylation with Other Reagents. Alkylation with IAA was generally used to prevent reoxidation of sulfhydryl groups exposed by disulfide reduction. IAA was added directly to the reduction medium at 25° (Waxdal *et al.*, 1968) in an amount which gave 2.5–5 mol of IAA/mol of DTT originally

TABLE I: Molecular Weights of Acetylcholinesterase Polypeptides from SDS Gels.[a]

Nonreduced[b]		Reduced[c]	
Peak	Mol Wt	Peak	Mol Wt
1	290,000	I	75,000
2	230,000	II	50,000
3	150,000	III	27,000
4	70,000	IV	23,500

[a] Molecular weights were determined by gel electrophoresis in 1% SDS as outlined in Methods. [b] Nonreduced samples were incubated without DTT. The molecular weights were estimated by reference to a standard curve for 3.5% gels which included covalently cross-linked ovalbumen (see Methods). Plots of the log mol wt *vs.* electrophoretic mobility were linear from molecular weights of 20,000 through the ovalbumen hexamer at 260,000. [c] Reduced samples were incubated with DTT as described in Methods. The molecular weights were estimated from a standard curve for 5.8% gels containing eight known proteins; the plot was linear from 20,000 to 92,500. The standard deviation of the known molecular weights from the best fit straight line was 4.2%. A similar standard deviation was observed for the acetylcholinesterase unknown molecular weights among four calibration runs.

TABLE II: Molecular Weights of Reduced Acetylcholinesterase Polypeptides from Column Chromatography in 6 M Guanidine Hydrochloride.[a]

Peak	Mol Wt
I	69,000
II	51,000
III	21,000[b]
IV	

[a] Molecular weights were determined by chromatography on Sepharose 6B in 6 M guanidine hydrochloride as outlined in Methods. The molecular weights were estimated from a standard curve for eight known proteins between 23,000 and 92,500. The curve deviated slightly from linearity above 50,000 (Fish *et al.*, 1969), and the standard deviation of the known molecular weights from the estimated best fit curve was 6.6%. A somewhat smaller standard deviation was observed for the acetylcholinesterase unknown molecular weights among two to three calibration runs. [b] Peaks III and IV could not be resolved adequately for accurate individual determinations. The listed value corresponds to samples containing approximately equal amounts of III and IV (*e.g.*, fraction 43, Figure 5).

added. After 20–60 min in the dark, the alkylated sample was subjected to electrophoresis or chromatography. No attempt was made to quantitate the amount of IAA reacting with the protein. Alkylation with NEM was used occasionally as a control to the alkylation with [^{14}C]NEM described above. The reaction was carried out by the addition of an excess amount of NEM at 25° for 20–60 min.

Cyanogen Bromide Cleavage. Sample polypeptides were cleaved with CNBr in a procedure similar to that of Waxdal *et al.* (1968). Lyophilized polypeptides (0.2–1.0 mg) were dissolved at a concentration of 8–10 mg/ml in a 70% formic acid solution containing 0.1% CNBr. The solution of CNBr in formic acid was prepared immediately before use. After allowing the sealed reaction mixtures to stand at room temperature for the indicated period, the reaction was terminated by the addition of 30–40 volumes of water followed by immediate lyophilization. Work with [^{14}C]NEM-labeled reduced acetylcholinesterase indicated that some label was cleaved under these conditions and appeared in the solvent volume of subsequent chromatographic runs. This cleavage required the presence of CNBr and was not observed in control incubations containing only 70% formic acid.

Fluorescent Labeling of CNBr Fragments. The procedure used the reagent Fluoram and generally followed Böhlen *et al.* (1973). The use of this reagent resulted in much higher polypeptide labeling than that obtained in analogous procedures (*e.g.*, Gray, 1967) with dansyl chloride. The lyophilized sample of CNBr fragment (200–500 μg of protein) was dissolved in 50 μl of 0.2 N NaOH and immediately neutralized with the mixture 25 μl of 0.4 N HCl and 25 μl of 0.4 M sodium phosphate buffer (pH 7.0). A 100-μl aliquot of Fluoram in acetone (2 mM) was added and allowed to stand at 25° for 10 min. Ultrapure guanidine hydrochloride (0.3 g) was dissolved in the sample solution followed by a second 100-μl aliquot of Fluoram in acetone (2 mM). The final solution (0.5 ml) was added directly to the analytical Sepharose 6B column equilibrated with 6 M guanidine hydrochloride. The fluorescence of the recovered fractions was read immediately after the completion of the column, because a slow decay of the fluorescence intensity was observed with time.

Calculation of Protein Recoveries of CNBr Fragments. Extinction coefficients for polypeptides I, II, and (III + IV) were calculated from the column fractionation in Figure 5. Assuming (1) $E_{280\,nm}$ (1%) of I is the same as that of the total enzyme in 6 M guanidine hydrochloride (16.6); (2) the ^{32}P cpm attached to protein is directly proportional to the moles of either I or II; (3) molecular weights of 70,000 (I), 50,000 (II), and 20,000 (III) (see Tables I and II); and (4) the polypeptide composition of I is the sum of II + III, one may calculate $E_{280\,nm}$ (1%) values of 13.1 for II and 25.2 for III (also assume 25.2 for IV). Using these extinction coefficients and the observed ^{14}C cpm in each polypeptide fraction, the following ratios of ^{14}C cpm/μg of protein were calculated from data in Figure 5 (standard deviations included): 306 ± 8 (I); 328 ± 6 (II); 310 ± 22 (III, IV).

Normalization of Fluorescence for CNBr Fragments from Equimolar Pools of Polypeptides I, II, and III. Two sources of fluorescence (F) variation among the equimolar fragment pools were (1) intrinsic differences in F resulting from a nonrandom distribution of amine groups between II and III; (2) experimental differences in F resulting from varying efficiencies in labeling a given concentration of amine groups. The first source was observed to be negligible for the three pools in Figure 6 in the following way: the ratio of the relative fluorescence, $F/\mu g$, for a common CNBr peptide in pools I and II (peptide between 65 and 75 g in Figure 6) between pools I and II was the same as the ratio of the (total F recovery)/(total μg recovery) between pools I and II. Hence all the variation in $F/\mu g$ must derive from the second source. A normalization factor for this source for either II or III + IV is simply the ratio of the (total F recovery)/(total μg recovery) between that for I and that for either II or III + IV.

Polyacrylamide Gel Electrophoresis in SDS. Initial experiments were performed according to procedures which utilize buffers containing 0.1% SDS during electrophoresis (Shapiro

et al., 1967; Weber and Osborn, 1969). Later work was carried out according to Fairbanks *et al.* (1971), in which 1.0% SDS is present during electrophoresis; this procedure, applied to gels either 5.8 or 3.5% in total acrylamide, was used to obtain all gel electrophoretic patterns and molecular weight estimates and for all protein and carbohydrate (PAS) staining in this report. Both the 5.8 and 3.5% gels contained the same ratio of bisacrylamide to acrylamide as that in Fairbanks *et al.* (1971). Non-glycoprotein controls were included in all PAS staining procedures to ensure that a positive reaction was not an artifact of SDS.

A wide variety of electrophoresis sample preparations utilizing varying conditions of protein denaturation was investigated in the initial experiments (Shapiro *et al.*, 1967; Weber and Osborn, 1969; Lenard, 1970; Swank and Munkres, 1971). The sample preparation medium of Fairbanks *et al.* (1971) (1% SDS; 6% sucrose; 10 mM Tris-Cl (pH 8); 1 mM EDTA (pH 8); 40 mM DTT), with an increase in the pyronin Y tracking dye concentration to 40 μg/ml, was used in conjunction with their electrophoresis procedure for all SDS gel molecular weight estimates in this report. A similar sample preparation medium was used in all other SDS gel experiments presented, with the exception that the concentration of DTT, the addition of alkylating agents, and the sample incubation time and temperature were varied in certain acetylcholinesterase samples as noted. For a given state of disulfide reduction (see Results), little variation in the relative acetylcholinesterase banding patterns was observed among these various sample preparation procedures.

Molecular weights of unknown polypeptides were estimated from their electrophoretic mobilities relative to polypeptide standards of known molecular weight (Shapiro *et al.*, 1967; Weber and Osborn, 1969; Fairbanks *et al.*, 1971). Ovalbumen was cross-linked with dimethyl suberimidate (Davies and Stark, 1970) according to the procedure of Carpenter and Harrington (1972).

Gel electrophoresis of cyanogen bromide fragments was carried out in 12.4% total acrylamide gels containing a 1:30 bisacrylamide/acrylamide ratio. The electrophoresis and sample preparation procedures were the same as those referred to above (Fairbanks *et al.*, 1971) except that the electrophoresis solvent included 8 M urea (Swank and Munkres, 1971). Electrophoresis was continued for 17 hr at 1 mA/gel tube.

SDS gel electrophoresis in both analytical and preparative procedures were carried out with the Buchler 3-1750 Polyanalyst apparatus in conjunction with a Lambda LPD 425 FM regulated power supply. Electrophoresis with the Fairbanks *et al.* (1971) procedure was carried out at 8 mA/tube for about 2 hr. Gels were polymerized in 6-mm i.d. glass tubes and ranged from 80 to 120 mm in length among various experiments. Gels were scanned at 280 nm with the ISCO Model 659 gel scanning attachment to the UA-4 absorbance monitor or at 560 nm with the Gilford Model 2410 S gel scanning attachment to the 2400 spectrophotometer.

Isolation of Polypeptide Components from SDS Gels. Sections of preparative gels containing polypeptide bands were removed in single slices immediately after electrophoresis. The location of the bands was suggested by the staining patterns observed after the electrophoresis of similar samples and confirmed by scanning the unstained preparative gel immediately after electrophoresis at 280 nm. Corresponding slices of identical preparative gels were pooled, and the polypeptide was extracted by homogenization of the slices with a Virtis "75" homogenizer at 25–50% maximum speed for 1 min in a 10-ml homogenization flask. About 2 volumes of distilled water were added with 1 volume of gel prior to homogenization and 2 to 3 volumes of water was used in transferring the homogenate to plastic centrifuge tubes after homogenization. Foaming was observed during homogenization under these conditions, but no foam left the homogenization flask. The transferred homogenate was spun at 20,000*g* for 10 min and the clear supernatant was removed. About two-thirds of the protein in each band was recovered in the supernatant. The supernatants were dialyzed against water at 25° overnight (with a water change after 4 hr), distributed into aliquots, and lyophilized. Overall recovery after lyophilization was about 50%.

Quantitative Gel Slicing. Gels containing radio-labeled components were sliced after incubation of the freshly run gels in 10% Cl_3CCOOH and 10% glycerol for 1.5–4.5 hr. Slicing was carried out with an MRA gel slicer after freezing the gels (Weinberg *et al.*, 1967); gel slices were varied quantitatively from 1 to 3 mm. Gel slices were prepared for scintillation counting in the usual fashion, except that for ^{32}P-labeled samples the incubation in NCS was carried out at 25°.

Chromatography on Agarose Gels in 6 M Guanidine Hydrochloride. Chromatography in unbuffered 6 M guanidine hydrochloride was used for both preparative and analytical purposes. Molecular weights of unknown polypeptides were estimated from their elution weights relative to those of polypeptide standards of known molecular weight (Davison, 1968; Fish *et al.*, 1969). These estimates were obtained from multiple runs on a single Sepharose 6B chromatography column (Beckman; 0.9 × 160 cm). The column was operated at 25° with a 50-cm hydrostatic pressure differential maintained by a 250-ml Marriott flask in all runs. The column flow rate was about 2.0 ml/hr for initial runs and 1.5 ml/hr for later runs. Fractions of 0.8–2.0 g were collected and maintained at 4°. Elution volumes (V_e) corresponded to elution of 50% of the polypeptide peak; only slight peak asymmetry due to trailing was observed. Polypeptide elution was monitored by the absorbance at 280 nm for most protein standards; by the absorbance at 407 nm for cytochrome *c;* and by scintillation counting of [^{14}C]NEM-labeled acetylcholinesterase polypeptides and [^{125}I]-α-bungarotoxin. The elution volume of the solvent (V_i) was monitored either by the absorbance at 280 nm of oxidized DTT in the sample onput or by scintillation counting of $^{22}NaCl$ (Amersham-Searle) added to the sample onput.

Graphical analysis of the molecular weight as a function of the elution volume has generally been carried out by way of the distribution coefficient K_d, defined as $K_d = (V_e - V_0)/(V_i - V_0)$ where V_0 is the column void volume; K_d is assumed to be approximately proportional to the log of the molecular weight (Fish *et al.*, 1969). It is inconvenient to measure V_0 for every run, since the use of blue dextran to estimate V_0 results in a tailing of blue dextran absorbance through the elution volumes of the polypeptides. This problem can be overcome by using one of the polypeptides itself as an indicator of the solvent volume external to the gel matrices. If the elution volume of such a reference polypeptide is termed V_e' and its distribution coefficient K_d', a second distribution coefficient function K_r may be defined as $K_r = (K_d - K_d')/(1 - K_d') = (V_e - V_e')/(V_i - V_e')$. It is apparent that K_r is proportional to K_d if K_d' is constant; consequently K_r may be plotted *vs.* the log of the molecular weight with as much justification as K_d. Estimates of polypeptide weights in this report arise from standard curves using K_r in which cytochrome *c* is the reference polypeptide with a $K_d' = 0.46$. Cytochrome *c* is convenient both because its molecular weight does not overlap with acetylcholinesterase polypeptides and because its absorbance may be unambiguously detected at 407 nm in the presence of overlapping polypeptide standards.

Protein samples for analytical column chromatography in

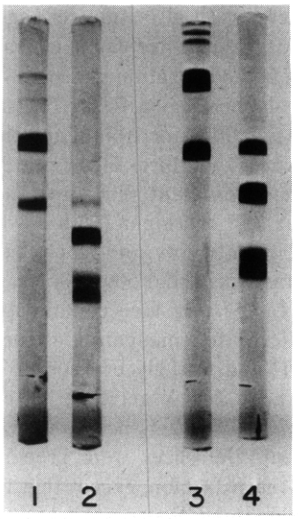

FIGURE 1: SDS gel electrophoresis of disulfide-reduced and nonreduced acetylcholinesterase: (1 and 2) 20 μg of preparation 3 after 6 months of storage (see Methods) was applied to each 3.5% acrylamide gel; (3 and 4) 50 μg of preparation B after 16 months of storage was applied to each 5.8% acrylamide gel. Samples were prepared in identical fashion by incubation in a solution containing 1% SDS at 37° for 2 hr (Fairbanks *et al.*, 1971) except that the disulfide-reduced samples 2 and 4 included 40 mM DTT while in samples 1 and 3 the DTT was deleted.

guanidine hydrochloride were prepared under conditions assumed to result in complete disulfide reduction. Protein standards (1–2 mg) were dissolved either in 0.2 ml of ultrapure 6 M guanidine hydrochloride or in 0.2 ml of column grade 6 M guanidine hydrochloride containing a ^{14}C-labeled acetylcholinesterase polypeptide. Addition of 50 μl of buffered DTT in 6 M guanidine hydrochloride to final concentrations of 40 mM DTT and 200 mM Tris-Cl (pH 8.5) was followed by incubation at 50° for 2 hr. Solid IAA was added to alkylate reduced protein as described above, and the sample was introduced manually to the resin upper surface.

Isoelectric Focusing. Experiments were performed on a vertical column (LKB 8100, 110 ml) utilizing carrier ampholytes (LKB-Producten) in a continuous sucrose density gradient (Haglund, 1971). The acetylcholinesterase samples were introduced in the light gradient solution and the anode was placed in the lower acid electrode solution (Instruction Manual, LKB 8100 Ampholine electrofocusing equipment (1968), p VIII:1). The addition of 6 M urea to all electrode and gradient solutions was made to increase the solubility of the acetylcholinesterase subunit polypeptides; a stock 6 M urea solution was deionized for 30 min with Bio-Rad AG 501-X8 (D) mixed bed resin to a conductivity of less than $0.5 \times 10^{-5}\ \Omega^{-1}\ cm^{-1}$ at 20° immediately before preparing the column solutions (Ui, 1971).

Preliminary experiments were carried out in 1% ampholine, pH 3–10, with about a 1-mg acetylcholinesterase sample. Since virtually all the recovered protein fluorescence in these preliminary experiments was found between pH 5.0 and 6.0 (uncorrected for 6 M urea), an ampholine fractionation run was conducted in 8% ampholine, pH 3–6, with about a 1-mg acetylcholinesterase sample. Fractions between pH 4.9 and 6.5 (uncorrected for 6 M urea) contained the enzyme and were pooled. Final electrofocusing profiles were obtained with the use of this ampholine pool diluted to a final concentration of 1%; in addition a 10-ml layer of 2% stock ampholine, pH 3–10, was placed between the sample solution and the basic electrode solution to minimize protein retention at the electrode solution interface (Chen *et al.*, 1974). Final profiles were obtained after electrofocusing at a constant voltage of 400 V for 60 hr at 4°.

Other Methods. Scintillation counting was carried out with 24-μl sample aliquots plus 100 μl of water which were incubated in vials containing 1 ml of NCS (Amersham) for 1 hr at 50°. After cooling, 10 ml of toluene containing 50 mg of 2,5-diphenyloxazole and 3 mg of dimethyl 1,4-bio[2-(5-phenyloxazolyl)]benzene (Packard) was added. Counts were recorded with a Packard Model 574 scintillation counter.

Fluorescence was monitored on an Aminco-Bowman spectrofluorometer.

Results

Polypeptide Composition. The polypeptide banding patterns resulting from the electrophoresis of 11S acetylcholinesterase in polyacrylamide gels containing 1% SDS are shown in Figure 1. Different sets of multiple bands are observed before and after disulfide reduction of the protein samples, and all the observed bands stain positively both for protein and for glycoprotein. As outlined in the introduction, the homogeneity of the native acetylcholinesterase preparations described in this report has been carefully studied; while the absence of contaminant protein has been confirmed, the relative amounts of the bands apparent after reduction varies with the preparation. All the bands observed in the SDS gel electrophoresis patterns would thus appear to correspond to components in a native 11S acetylcholinesterase molecule, and further confirmation of this point is given in the ^{32}P-labeling patterns below.

The apparent molecular weights of the polypeptide components estimated from gel electrophoresis in SDS are given in Table I, and those from chromatography in 6 M guanidine hydrochloride are given in Table II. The four bands apparent after disulfide reduction in these gels are labeled I–IV and decrease in molecular weight from 75,000 to 23,500. Four species equal to or larger than I are observed prior to reduction, with the major component corresponding to a molecular weight of about 150,000. The molecular weights of the four nonreduced components were estimated from a standard curve which included covalently cross-linked ovalbumen. Some variation in the relative mobilities of the ovalbumen oligomers was observed among cross-linked preparations, so the molecular weight estimates for the nonreduced acetylcholinesterase species were not as precise as those for the reduced species. Nevertheless, these estimates indicated relative molecular weights which were simple multiples of the smallest nonreduced species regardless of which standard curve was used. This relationship would suggest that the three larger nonreduced species are oligomeric forms of the smallest, and the ^{32}P-labeling patterns below are consistent with this formulation. The molecular weights of I and II estimated from SDS gels are somewhat lower than those reported by other workers for the major bands observed after disulfide reduction of the 11S species (Dudai and Silman, 1971; Berman, 1973; Powell *et al.*, 1973). These other reports were based on gel electrophoresis in 0.1% SDS. Our initial experiments in 0.1% SDS gels gave estimated molecular weights for I–IV which were 5–10% higher than those in Table I. This discrepancy in relative electrophoretic mobility may reflect a reduced affinity of SDS for acetylcholinesterase resulting from its glycoprotein nature (Segrest *et al.*, 1971); higher estimated molecular weights were also observed in 3.5% acrylamide gels, presumably for the same reason. The estimates from 1.0% SDS gels correspond more closely to molecular weight estimates from column chromatography in 6 M guanidine hydrochloride (also see Fairbanks *et al.*, 1971).

Labeling with [^{32}P]DFP. The susceptibility of all the observed polypeptide components to relatively irreversible acyla-

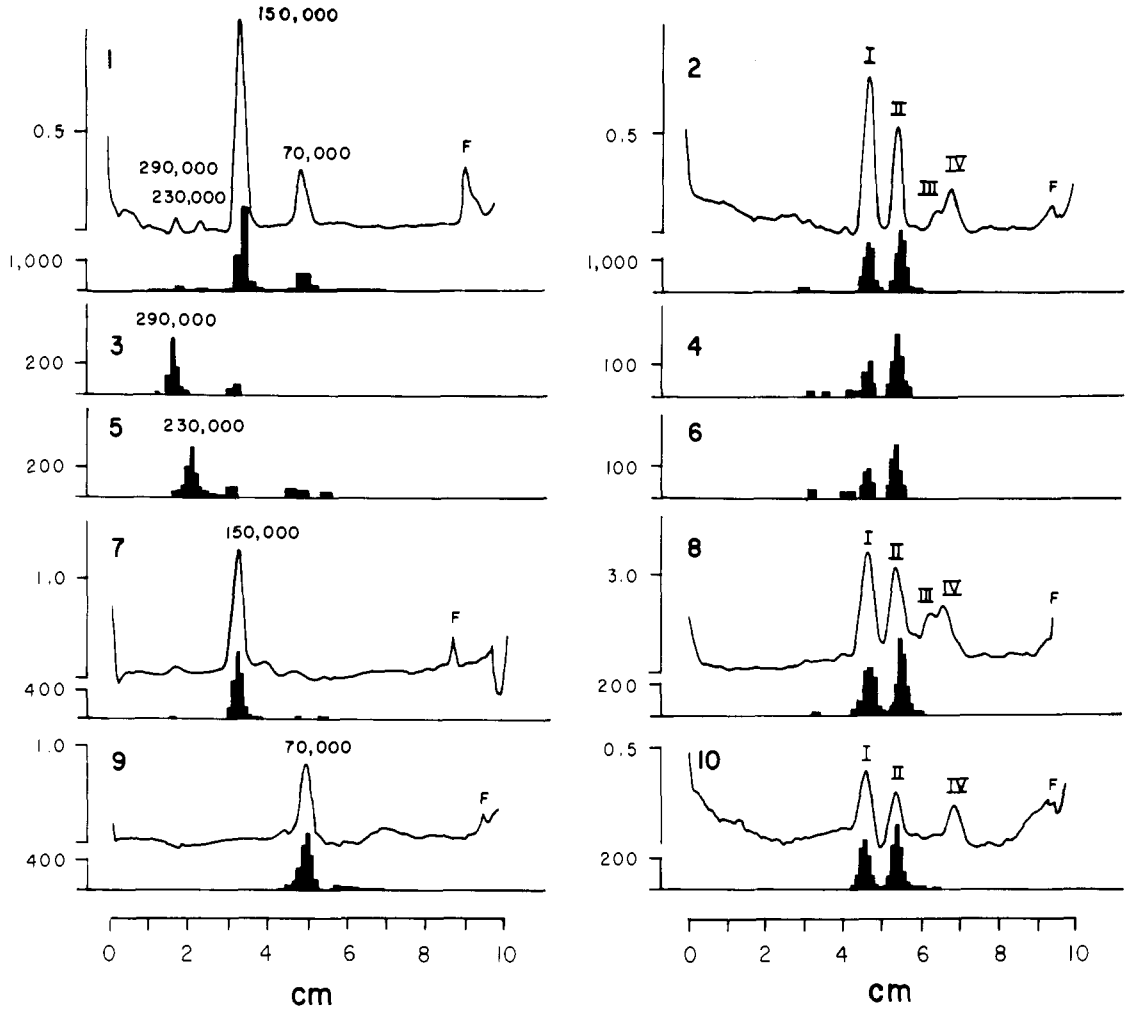

FIGURE 2: [^{32}P]DFP labeling of polypeptide components in acetylcholinesterase as observed on SDS gels. Samples of 55 μg of nonreduced [^{32}P]DIP-acetylcholinesterase (preparation 4 after 6 months of storage) were run on each of four preparative 3.5% gels and the polypeptide components were isolated as outlined in Methods. Portions of each isolated component were then run after incubation for 1 hr at 50° in the usual sample preparation medium containing either Tris-Cl buffer (20 mM, pH 8.5) or buffer plus 40 mM DTT. Gels (3.5%) containing identical components were run in parallel and either quantitatively sliced or stained and scanned. The odd-numbered gel records refer to nonreduced polypeptide samples; the even-numbered gel records correspond to disulfide-reduced samples. Nonreduced polypeptide components are named according to their apparent molecular weights in Table I. The solid line is the absorbance scanned at 280 nm; the black histograms refer to the total ^{32}P cpm per 1-mm gel slice; the dye front is labeled F: (1 and 2) [^{32}P]DIP-acetylcholinesterase from the preparative gel sample pool; 12-μg onputs for sliced gels, 10-μg onputs for scanned gels; (3 and 4) 290,000 component; 1.0-μg onputs; (5 and 6) 230,000 component; 1.2-μg onputs; (7 and 8) 150,000 component; 3.0-μg onputs for sliced gels; (7) 12-μg and (8) 36-μg onputs for stained gels; (9 and 10) 70,000 component; 3.0-μg onputs for sliced gels; 7-μg onputs for stained gels.

tion with [^{32}P]DFP is shown in Figure 2. Acylation of acetylcholinesterase with DFP is generally assumed to occur only at the active site serine (Schaffer *et al.*, 1973); and the cyanogen bromide fragment patterns of [^{32}P]DIP-acetylcholinesterase described below show only a single ^{32}P-labeled fragment, in agreement with this assumption. All four components present prior to disulfide reduction are labeled, but only polypeptides I and II after reduction contain ^{32}P.

Several characteristics of the individual polypeptides may be derived from a detailed analysis of the data in Figure 2. Isolation of each of the nonreduced components and separate electrophoresis of the isolated species without disulfide reduction demonstrates that the size of each nonreduced component is relatively stable under the isolation conditions. After disulfide reduction, however, all four components are converted to closely similar patterns which account for the pattern seen on reduction of the unfractionated enzyme. In particular, the relative amounts of ^{32}P-label associated with polypeptides I and II arising from reduction are the same, within 8%, for each of the four nonreduced components and the unfractionated enzyme. For the 70,000, 150,000, and total enzyme species in runs 2, 8, and 10, the ratio of ^{32}P in I to ^{32}P in II is 0.81 $\pm$ 0.01. While the two minor nonreduced components which correspond to the 230,000 and 290,000 species cannot be isolated in sufficient quantity to scan their stained polypeptide composition, scans of the gels containing the 70,000 and 150,000 species indicate no additional stained bands prior to disulfide reduction and only polypeptides I–IV after reduction. The scans of the polypeptide composition of the 70,000 and 150,000 species after reduction do indicate one interesting difference; nearly equal amounts of III and IV appear present in the reduced 150,000 species, but only IV is observed in the reduced 70,000 component. A suggested origin of this difference is discussed below. Since other data indicate that IV is derived from III (T. L. Rosenberry, manuscript in preparation, and see Figures 7 and 8), nearly all the protein apparent in the 150,000 species can be accounted for in the polypeptide composition of the reduced 70,000 species. This result plus the 2:1 ratio of the apparent molecular weights provide strong evidence that these species represent a dimer–monomer relationship involving closely similar 70,000

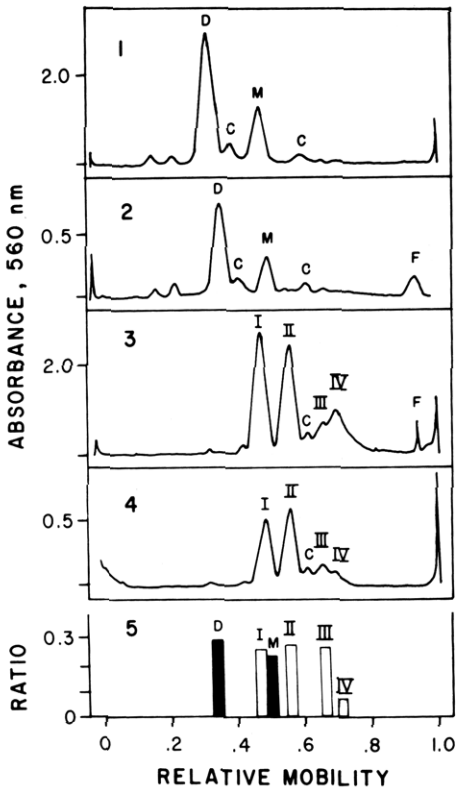

FIGURE 3: Relative staining intensities of 3.5% acrylamide gels containing the polypeptide components of acetylcholinesterase after electrophoresis in SDS and staining either with Coomassie Blue or with the PAS procedure. Stained gels were scanned at 560 nm. The outlines of each peak were traced and the relative areas were determined from the weight of the traces. Samples of acetylcholinesterase (preparation 4 after 11 months of storage) were either nonreduced (1 and 2) or reduced (3 and 4) as outlined in Figure 1: (1 and 3) 20 μg, Coomassie Blue stain; (2 and 4) 100 μg, PAS stain; (5) histograms of the ratios of the PAS absorbance peak area to the Coomassie absorbance peak area for each of the major components; black, nonreduced gels; white, reduced gels. The peaks are identified as the following components: D, dimer, M, monomer, from nonreduced samples; C, apparent degradation product of acetylcholinesterase which arose during preparation of these samples; F, dye front. The slightly greater mobility of components in the PAS-stained gels arises from the high sample load.

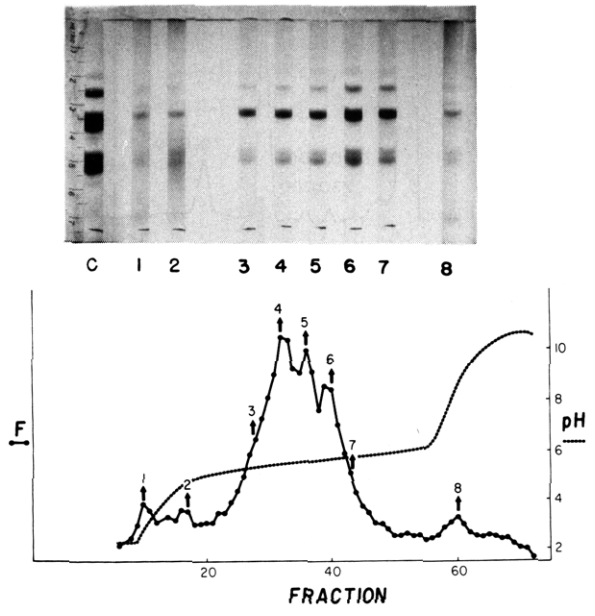

FIGURE 4: Isoelectric focusing of acetylcholinesterase in 6 M urea with a prefractionated 1% ampholine pool, pH 5–6, containing 10 mM DTT. Details are described under "final electrofocusing profiles" in Methods. The sample was 3 mg of stock enzyme (preparation 3 after 3 months of storage) which had been reduced (6 M urea–0.5 M Tris-Cl–20 mM DTT (pH 8.5) for 1 hr at 40°) and dialyzed against 6 M urea–10 mM DTT. The protein fluorescence *F* (excitation 290 nm, emission 350 nm) was monitored for each fraction. Indicated fractions were dialyzed extensively against water and lyophilized. Lyophilizates were prepared for SDS gel electrophoresis with DTT as in Figure 1: Gel onputs (C), 50-μg control enzyme; (1, 2, 6–8) total lyophilizate; (3) $\frac{2}{3}$ total lyophilizate; (4 and 5) one-half total lyophilizate.

subunits. Since the major species prior to disulfide reduction is the dimer, it appears that a covalent intersubunit linkage which involves disulfide bonding is present in this species; this point is examined in detail elsewhere (T. L. Rosenberry, manuscript in preparation). The two nonreduced minor components of higher molecular weight presumably represent trimer and tetramer forms of the monomer subunit.

Carbohydrate Content. The glycoprotein nature of eel acetylcholinesterase was suggested from the presence of hexosamines in the amino acid hydrolysates (Leuzinger and Baker, 1967) and confirmed by sialic acid analyses of the isolated polypeptides (Powell *et al.*, 1973). The relative amounts of carbohydrate associated with each of the observed polypeptides was estimated from SDS gel electrophoresis profiles which had been stained by the PAS procedure. Scans of these gels and control gels stained for protein with Coomassie Blue are shown in Figure 3. The relative carbohydrate content of each band was estimated by calculating the ratio of the areas of the PAS stained to the Coomassie stained peaks. While this procedure should be considered only semiquantitative, it is apparent that a significant deviation in this calculated ratio occurs in polypeptide IV, which has a much lower ratio than the other polypeptides. The total area of the PAS positive peaks was the same in gels containing either reduced or nonreduced samples; this area corresponded to 1.7 times that of an equivalent load of horse transferrin, a glycoprotein containing 6.5% carbohydrate (Hudson *et al.*, 1973). PAS positive reactions of about the same intensities as those in Figure 3 were observed on SDS gels of the individual polypeptide components after the fractionation shown in Figure 5 below.

Fractionation of Disulfide-Reduced Polypeptides. It was noted above that variations in the relative polypeptide compositions after reduction among several 11S acetylcholinesterase preparations suggest that smaller polypeptides derive from cleavage of a single 70,000 subunit (Chen *et al.*, 1974). To confirm this suggestion, efforts to fractionate and characterize the individual reduced polypeptides were initiated. Isoelectric focusing in 6 M urea containing 10 mM DTT was investigated as a fractionation technique; Figure 4 shows an electrofocusing profile of acetylcholinesterase under these conditions. SDS gels of selected fractions are also shown in Figure 4 to characterize the polypeptide composition. No significant fractionation of the reduced polypeptides was achieved; either the reduced polypeptides do not dissociate in 6 M urea, or all four components have apparent pK_I's of 5.5 ± 0.2. If this value is adjusted for the effect of urea (Ui, 1971), the pK_I's become 5.1 ± 0.2. Ion exchange chromatography in 6 M urea with DEAE-cellulose (Whatman DE 52) also failed to give significant fractionation of the reduced polypeptides.

A partial fractionation adequate for several studies was achieved by preparative Sepharose 4B chromatography in 6 M guanidine hydrochloride. Figure 5a shows the fractionation pattern achieved with [^{14}C,^{32}P]DIP-acetylcholinesterase which had been reduced with DTT, alkylated with [^{14}C]NEM, and introduced to the column without dialysis. In addition to the column elution profile, SDS gel electrophoresis patterns of selected fractions from the column are shown in Figure 5b. Poly-

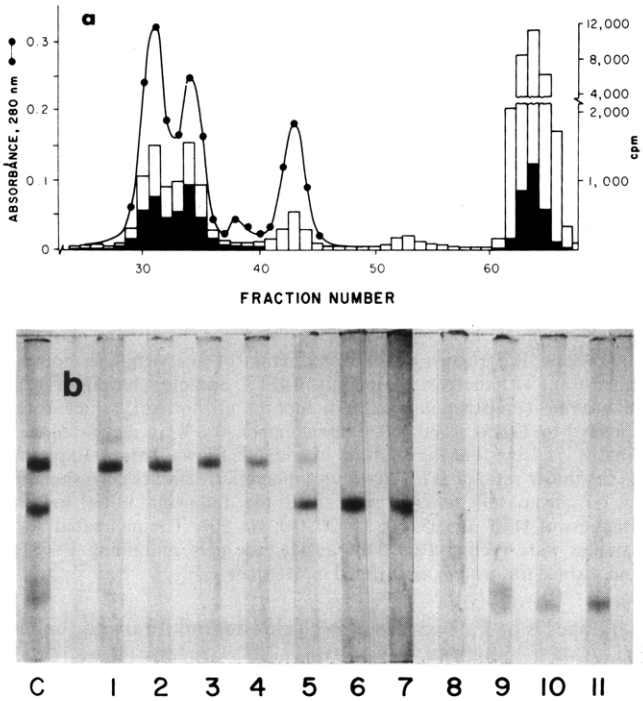

FIGURE 5: (a) Fractionation of disulfide-reduced [^{14}C,^{32}P]DIP-acetylcholinesterase which had been alkylated with [^{14}C]NEM on a Sepharose 4B column equilibrated with 6 M guanidine hydrochloride. The preparation of the 25-mg sample of [^{14}C,^{32}P]DIP-acetylcholinesterase is given in Methods. The sample was introduced to the bottom of a Sepharose 4B column (2.5 × 85 cm) which was linked in tandem to a second Sepharose 4B column (2.5 × 60 cm); both adapter-fitted columns (Pharmacia) were previously equilibrated with 6 M guanidine hydrochloride and were operated with an upward flow of 7.5 ml/hr maintained with hydrostatic pressure. The average fraction size was 13 g (solvent weight). The total area under the white histogram corresponds to the ^{14}C cpm of a 25-μl fraction aliquot; the area under the black histogram refers to the ^{32}P cpm of the same aliquot. (b) SDS gel electrophoresis of selected fractions from the column in Figure 5a. Aliquots of fractions which corresponded to 0.036 (OD_{280}) (ml) (22 μg according to $E_{280\,nm}$ (1%) 16.6) were dialyzed against water extensively and lyophilized. Lyophilizates were dissolved in 50 μl of the 1% SDS sample solvent minus DTT (see Figure 1) and 25 μl was added to each gel: (C) column onput control, 22 μg; (1 and 11) fractions 29, 30, 31, 32, 33, 34, 35, 38, 42, 43, 44.

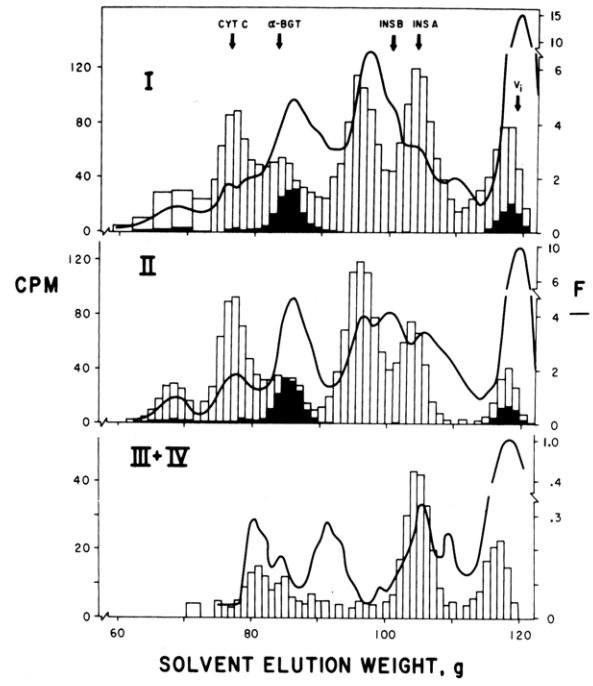

FIGURE 6: Cyanogen bromide fragment composition of isolated acetylcholinesterase polypeptides as observed by chromatography in 6 M guanidine hydrochloride. Aliquots of fractions 31 (I, 2.5 ml), 34 (II, 2.5 ml), and 43 (III + IV, 2.0 ml) from the preparative column in Figure 5 were each dialyzed extensively against water and lyophilized. CNBr cleavage was carried out for 2 hr, the fragment samples were fluorescent labeled, and the samples were applied to the analytical 6B column equilibrated in 6 M guanidine hydrochloride as indicated in Methods. Protein fragment recoveries were 341 μg (I), 228 μg (II), and 48 μg (III + IV), calculated from the total ^{14}C recoveries as described in Methods. These amounts were 35–70% of the corresponding ^{14}C pools taken for dialysis. *F*, relative fluorescence (excitation 390 nm, emission 480 nm); the white histograms are ^{14}C cpm, the black histograms are (0.8) (^{32}P cpm). The indicated elution weights of reduced and alkylated cytochrome *c*, and [^{125}I]-α-bungarotoxin, and fluorescent-labeled insulin A and B and V_i (^{22}NaCl) were from a separate calibrating run.

peptides I and II are adequately resolved; fractions of each component are obtained essentially free of contamination by the other polypeptides. Polypeptides III and IV are not resolved and are obtained as mixtures in various proportions. A region of low 280-nm absorbance between II and III is observed, but the corresponding SDS gel shows only a diffuse region of relatively low protein staining (also see Figure 3). A small peak containing ^{14}C label but no OD_{280} is eluted after IV. The origin of this labeled material is unclear; it appears associated with the native enzyme but is not a polypeptide which corresponds to the difference between III and IV (T. L. Rosenberry, manuscript in preparation). Both of these minor components may represent limited polypeptide cleavages occurring at sites other than those which give rise to the major polypeptide fragments described in this report.

Efforts to further resolve fractions of III and IV mixtures in 6 M guanidine hydrochloride were made on Sepharose 6B columns. The incomplete degree of separation achieved indicated a molecular weight difference between III and IV of less than the 3500 estimated from SDS gels.

Characterization of the Isolated Polypeptides. To examine the suggestion that II, III, and IV all derive from I by polypeptide cleavage more closely, CNBr fragments of the isolated ^{14}C,^{32}P polypeptide pools from the preparative column in Figure 5 were prepared. From the methionine content (Rosenberry *et al.*, 1972) one would expect 15–18 fragments from an intact polypeptide I. The fragments were fluorescent labeled and fractionated in the analytical Sepharose 6B column equilibrated in 6 M guanidine hydrochloride. Results of one set of experiments are shown in Figure 6; the amounts of each of the three labels associated with each fraction is given for fragments generated from I, from II, and from a mixture of III and IV. The patterns obtained with the three labels are consistent with the expected number of fragments. Only one major ^{32}P-labeled peak corresponding to a fragment with an apparent molecular weight of 7000 is observed for both I and II, in addition to a smaller amount of ^{32}P-hydrolysis product in the solvent elution volume V_i. Three major ^{14}C-labeled fragment fractions with elution weights of 77, 95, and 104 g are present in I in addition to a ^{14}C-degradation product at V_i (see Methods); numerous other fragments are indicated by the fluorescent Fluoram label attached nonspecifically to amine groups.

A quantitative comparison of the three CNBr fragment profiles is shown in Figure 7. The profiles have been normalized to correspond to equimolar amounts of polypeptides I, II, and III. It is apparent that the sum of the ^{14}C-labeled fragment profiles for II plus the III + IV mixture corresponds completely to that for I. A similar correspondence is seen for much of the fluorescence profiles; however, a fragment pool at 96 g in I appears to

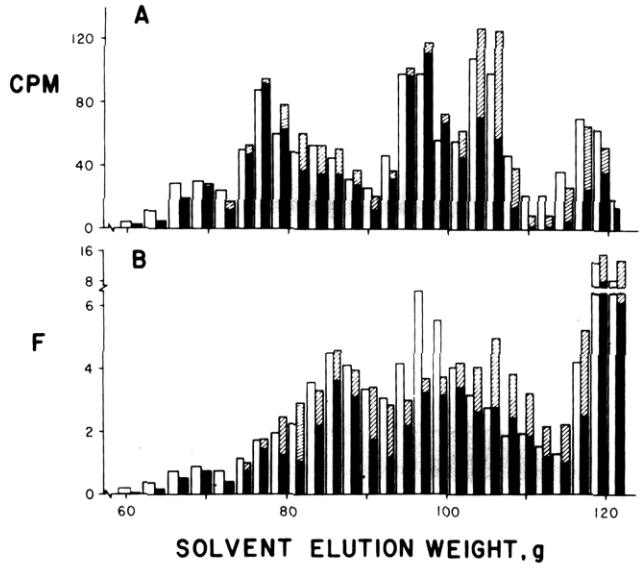

FIGURE 7: Comparison of cyanogen bromide fragment fractionation profiles and demonstration that the fragment composition of I is essentially the sum of the fragment composition of II plus the III + IV mixture. (A) ^{14}C cpm profiles in Figure 6 were normalized to correspond to equimolar pools of I, II, and III + IV with molecular weights of 70,000 (I), 50,000 (II), and 20,000 (III + IV) (see Tables I and II). The normalization factors were 1.00 (I), 1.07 (II), and 2.03 (III + IV); (B) *F* profiles in Figure 6 were normalized both as in (A) and as indicated in Methods. The overall *F* normalization factors were 1.00 (I), 0.85 (II), and 7.2 (III + IV). The normalized profiles are compared as histograms for each 2-g interval with I (□); II (■); and III + IV (▨).

be missing in the sum of II plus III + IV, and a broader fragment pool at 105 g in II plus III + IV is not present in I. This shift probably arises from a reduction in size of one or two fragments in I as a result of cleavages A and/or B which generates polypeptides II, III, and IV.

In the experiment in Figure 6, CNBr was incubated with the respective polypeptide pools for 2 hr; other experiments in which the incubation proceeded for 4 hr permitted the effect of the CNBr incubation time on the fragment composition to be evaluated. After 4 hr the minor ^{14}C-labeled fragments at 68 and 84 g completely disappear: the major ^{14}C fragments decrease only to the extent that additional ^{14}C-degradation prod-

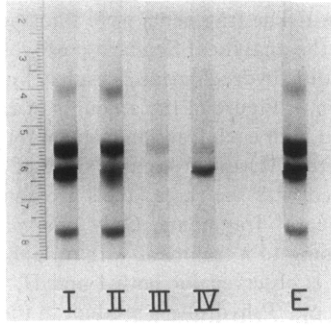

FIGURE 8: Cyanogen bromide composition of intact acetylcholinesterase and isolated polypeptide pools obtained from electrophoresis in SDS–urea. Reduced and alkylated enzyme (column onput, Figure 5, 1.75 mg) and 10-ml aliquots of fractions 30 (I), 35 (II), 41 (III), and 44 (mostly IV) from the preparative column in Figure 5 were each dialyzed extensively against distilled water and lyophilized. CNBr cleavage was carried out for 4 hr and small aliquots were lyophilized. The lyophilizates were redissolved in 25–45 μl of a solution containing 1% SDS and 10-μl portions were introduced for gel electrophoresis as outlined in Methods. Gel onputs: (I) 55 μg; (II) 52 μg; (III) 10 μg; (IV) 10 μg; (E, total enzyme) 55 μg. The cm scale at left indicates the relative mobility; the top of the gels are at 0 cm and the dye fronts are at 9.9–10.0 cm. All observed Coomassie-staining bands as shown in the photograph.

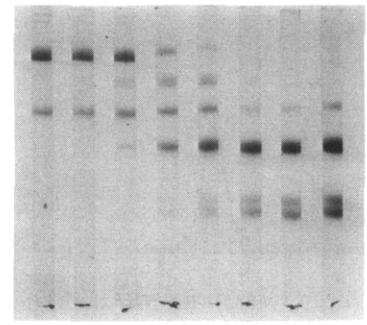

FIGURE 9: Progressive disulfide reduction of acetylcholinesterase in denaturant with increasing amounts of DTT. Sample solutions of 30 μg of enzyme (preparation 3 after 6 months of storage), which corresponded to 20 μM in 70,000 subunit monomers, were incubated as in Figure 1, except that the quantity of added DTT was varied. Expressed as the molar ratio of DTT to subunit monomer, samples corresponding to gels from left to right contained the following initial relative amounts of DTT: 0.5; 5; 10; 15; 30; 40; 50; 500. The incubated 20-μl samples were then run on 5.8% acrylamide gels containing 1% SDS and stained for protein as outlined in Methods.

uct appears at V_i. Because of both this degradation and the fact that only about 75% of the total ^{14}C-labeling capacity was achieved (T. L. Rosenberry, manuscript in preparation), it is difficult to quantitate the distribution of ^{14}C among the fragments in stoichiometric terms. These patterns and the relative ^{14}C cpm recoveries in Figure 5a are qualitatively consistent with an apparent value of about seven sulfhydryl groups in the totally reduced subunit monomer (T. L. Rosenberry, manuscript in preparation). The ^{14}C-degradation product also obscures a possible small ^{14}C fragment which may be present near V_i in I, III, and IV.

The CNBr fragment composition of individual pools of each of I, II, III, and IV (III and IV resolved from each other to about 75%) were compared with that of the total unfractionated enzyme by electrophoresis on 12% acrylamide gels in the presence of SDS and urea (see Methods). The gel patterns obtained are shown in Figure 8. While fewer polypeptide bands are observed after the staining procedure than would be expected from the compositions in Figure 6, essentially the same result is obtained: all components observed in the intact unfractionated enzyme are observed both in I and in the sum of II, III, and IV. Apparently some polypeptide components were washed from the gels during the staining and destaining procedures.

It is apparent that, for all three labels in the profiles in Figure 6 and for all five pools in Figure 8, the sum of the fragment compositions of II plus III + IV yields essentially the composition for I. Further evidence that IV is derived from III is obtained from sulfhydryl modification studies; under conditions in which only one sulfhydryl per subunit is labeled, the label appears only in I, III, and IV (T. L. Rosenberry, manuscript in preparation). The evidence is conclusive that II, III, and IV all are derived from I by polypeptide or glycopeptide cleavages and that I is equivalent to the intact monomer subunit.

Structural Relationship of the Subunit Polypeptides. Information about disulfide linkages between the component polypeptides was obtained from the experiment shown in Figure 9, where the extent of disulfide reduction in 1% SDS was controlled by limiting the amount of added DTT. The observed polypeptide which is released from the subunit dimer at the lowest DTT concentration is II. This 50,000 species arises simultaneously with a polypeptide doublet with molecular weights which are estimated to be 102,000 and 105,000. The molecular weights of the doublet thus correspond to I plus III

and I plus IV. At higher DTT concentrations the polypeptides comprising the doublet disappear among the smaller polypeptides I–IV. If the subunits are covalently linked in the dimer with a high degree of symmetry, it would appear that the portion of the intact subunit through which the linkage is maintained is within polypeptide IV.

Discussion

Subunit Structure. Our data on 11S acetylcholinesterase lead to the following conclusions. The active enzyme is a tetramer composed of subunits with molecular weights of 70,000. The subunits each contain one active site and are identical, within our limits of observation, except for a variability in manifesting two sites of cleavage, probably caused by proteolytic or glycolytic agents. This result is consistent with our previous reports that the 11S enzyme had at least 3.8 active sites per 280,000 daltons (Rosenberry *et al.*, 1972; Chen *et al.*, 1974) and that the active sites are kinetically homogeneous (Rosenberry and Bernhard, 1971). It also confirms an earlier suggestion by Kremzner and Wilson (1964). One cleavage occurs at a site which may be called A and splits the intact subunit, polypeptide I, into a major fragment, polypeptide II, and a minor fragment, polypeptide III. A polypeptide IV appears to be derived from III by a second cleavage, B; this relationship is discussed below. This cleavage scheme was initially indicated by the relative polypeptide compositions of several 11S enzyme preparations (Chen *et al.*, 1974); it has now been confirmed by the observation that the sum of the CNBr fragment compositions of II and a III,IV mixture yields the composition of I. The cleavages at A and B do not appear to result in the release of measurable polypeptides from the subunit prior to disulfide reduction, even in the presence of strong denaturing agents. The predominant species in 1% SDS prior to reduction is a subunit dimer, and clear evidence that a disulfide bond is involved in this linkage has been obtained (T. L. Rosenberry). A similar pattern has been reported in 0.1% SDS gels (Dudai and Silman, 1971). In addition, species with molecular weights corresponding to monomer, trimer, and tetramer are seen prior to reduction. A predominate species from the 11S enzyme with a molecular weight corresponding to a subunit dimer has also been reported in 5 M guanidine hydrochloride prior to disulfide reduction (Froede and Wilson, 1970).

Polypeptides I–IV are released separately after disulfide reduction in 1% SDS or 6 M guanidine hydrochloride. Labeling of the native enzyme with [^{32}P]DFP reveals that both I and II possess an active site. The appearance of bands similar to I and II under analogous conditions has been reported by a number of laboratories, both for the 11S enzyme species (Dudai and Silman, 1971; Berman, 1973; Powell *et al.*, 1973) and for the 14S and 18S species (Dudai *et al.*, 1972b; Powell *et al.*, 1973). The presence of two polypeptide chains after disulfide reduction of 11S enzyme in 8 M urea was initially reported by Leuzinger *et al.* (1969); the presence of two C-terminal amino acids in their enzyme preparation led these workers to suggest that the enzyme was a dimeric hybrid $(\alpha\beta)_2$, where α and β were the two polypeptides. While some later reports considered a proteolytic cleavage of a single subunit polypeptide to be a more likely explanation for the appearance of polypeptides I and II(Dudai *et al.*, 1972a; Bock *et al.*, 1973), other reports favored the dimeric hybrid model and equated I and II with α and β (Berman, 1973; Powell *et al.*, 1973). Our data unequivocally rule out such a dimeric hybrid model and instead support a tetrameric structure which is a dimer of dimers $((\alpha_2)_2)$, where α is either the cleaved or intact subunit containing the catalytic site; in particular, Figure 2 demonstrates that the polypeptide composition of the nonreduced dimer is essentially identical with that of the nonreduced monomer and does not consist of stoichiometric amounts of I and II. Our data also are incompatible with an interpretation of three dimer species reported recently for nonreduced 11S enzyme on 5% acrylamide gels containing SDS (Powell *et al.*, 1973); the molecular weights of such large protein species are difficult to estimate on 5% acrylamide gels, and the larger two of these reported dimers may correspond to the trimer and tetramer species which are quantitated more accurately on 3.5% gels.

The variable ratio of I to II found in several preparations of 11S enzyme in our laboratory (Chen *et al.*, 1974) offers an explanation for the highly diverse subunit molecular weights reported for the disulfide-reduced 11S enzyme in guanidine hydrochloride. Subunit molecular weights of 64,000 (Leuzinger *et al.*, 1969) and 42,000 (Millar and Grafius, 1970) from sedimentation equilibrium and 49,000 (Froede and Wilson, 1970) from sedimentation velocity experiments have been found. These techniques apparently are unable to distinguish the presence of two polypeptides with molecular weights as close as I and II. All these values fall within reasonable distance of 50,000 to 70,000, the extreme molecular weight values expected for averages of various mixtures of I and II.

Molecular Weight of the 11S Enzyme. The question of the molecular weight of 11S acetylcholinesterase has recently been raised by Dudai *et al.* (1973). These authors reported equilibrium sedimentation values of 350,000 ± 10,000 and 320,000 ± 10,000 for the 11S enzyme obtained, respectively, from trypsin-treated frozen and from toluene-stored electric eel tissue (Dudai *et al.*, 1972a). Their report differed significantly from the equilibrium sedimentation value of 260,000 ± 10,000 reported previously (Leuzinger *et al.*, 1969; Millar and Grafius, 1970). A slight but significant difference between the sedimentation coefficients of 11S preparations from different laboratories had also been seen previously (Massoulié *et al.*, 1970). The apparent variance in the molecular weight of the 11S species was suggested to reflect varying degrees of autolysis or proteolysis which could give rise to different preparations with different subunit sizes (Dudai *et al.*, 1973).

Preparations in our laboratory have demonstrated both a variability and a heterogeneity (Chen *et al.*, 1974) with respect to the extent of polypeptide cleavage at the discrete site A, but this cleavage does not appear to result in the loss of polypeptides from the subunit in the absence of disulfide reduction, as noted above. For the most extensively cleaved preparation we have seen (Figure 9), about 80% of the protein exists in the dimer form prior to disulfide reduction and may be progressively broken down to *discrete* smaller polypeptides with increasing disulfide reduction. The apparent molecular weight of the dimer in this preparation is the same as that in preparations with less cleavage. The discrete nature of the observed polypeptide bands and the sharpness and singularity of the minor bands corresponding to trimers and tetramers of the catalytic subunit prior to disulfide reduction indicate that the 11S preparations in our laboratory are homogeneous within our limits of observation with respect to the size of the native molecule. It is possible that proteolytic processes prior to the isolation of our purified enzyme have resulted in the quantitative loss of a well-defined polypeptide segment from all the subunits. Such a possibility cannot be excluded without a direct comparison of the various 11S preparations in question, but appears unlikely for two reasons; first, our SDS gel patterns from enzyme preparations which have relatively less cleavage appear similar to that from the trypsin-treated 11S enzyme preparation with the higher estimated molecular weights of both native tetramer

and SDS monomer (Dudai *et al.*, 1972a); and second, this estimate for the SDS monomer is apt to be somewhat high because of the technical difficulties (see Results) in estimating the molecular weights of glycoproteins on SDS gels. A second possibility which could give rise to variable enzyme molecular weight estimates for 11S enzyme involves residual material remaining attached to the 11S tetramer after transformation from 8S, 14S, or 18S species. If this transformation involves a degradation of a "tail" component seen on electron micrographs (see introduction) which is distinct from the catalytic subunits, varying amounts of residual "tail" component could be attached to 11S enzyme in different preparations. Such a residual component would be difficult to detect on SDS gels if it were heterogeneous.

Formation of Subunit Fragments. The occurrence of polypeptide fragmentation has been of concern in many reports of protein structure. Our observations of significant cleavage only at two discrete sites on the native acetylcholinesterase subunit appear to parallel similar observations on conconavalin A (Wang *et al.*, 1971), where cleavage occurs predominately on an exposed polypeptide loop without the release of significant fragments from the native molecule (Edelman *et al.*, 1972). Exposed sites frequently give rise to discrete cleavage on incubation with proteolytic agents (*e.g.*, ribonuclease S, Richards and Vithayathil, 1959). It is not clear that the discrete cleavage of acetylcholinesterase occurs at such exposed sites, since extensive incubation of the native enzyme with trypsin does not alter the polypeptide composition (Dudai *et al.*, 1972a). This observation plus the fact that some cleavage at site A has occurred in all polypeptide patterns reported for eel acetylcholinesterase indicate that this cleavage may be nontrivial and of possible physiological significance (see Chen *et al.*, 1974).

The relationship between cleavage at sites A and B appears somewhat complex. The relative amounts of II, III, and IV found in several purified acetylcholinesterase preparations were the same despite wide variations relative to I (Chen *et al.*, 1974). This observation indicates that cleavage B, generating IV from III, either (1) does not occur or (2) is not revealed prior to cleavage A. To maintain the observed constancy in the relative amounts of II, III, and IV, the first possibility requires the following scheme. Cleavage B would be rate-limited by

$$\mathrm{I} \xrightarrow[\text{slow}]{\mathrm{A}} \mathrm{II} + \mathrm{III}$$

$$\mathrm{III} \xrightarrow[\text{fast}]{\mathrm{B}} \mathrm{IV} + \mathrm{V}$$

prior cleavage at A. The failure of cleavage B to go to completion with quantitative formation of IV from III is discussed below. The second possibility arises because the difference between III and IV, a small segment V of perhaps 1000 to 3000 not directly observed in our procedures, is rich in and may be composed entirely of carbohydrate (Figure 3). Hence our reluctance to call cleavage B a proteolytic or polypeptide cleavage. Cleavage B is detected as the difference either between III and IV or between the subunit plus III and the subunit plus IV (Figure 9); in both cases the release of II from a subunit cleaved at A may expose the carbohydrate-rich section V. Prior to this exposure either V would not be released from the subunit or the mobility of polypeptides lacking V would not be measurably different on SDS gels from those possessing it.

A reason for the failure of cleavage at site B to completely convert III to IV is suggested by the reduced polypeptide composition arising from isolated subunit dimers and subunit monomers (Figure 2). The isolated dimer fraction includes cleaved subunits which contain approximately equal amounts of III and IV, whereas cleaved subunits from the isolated monomer fraction contain only IV. This result implies that only one-half the subunits involved in the disulfide cross-linked dimer are susceptible to cleavage at site B. The remaining subunits appear to be protected by a process which involves negative cooperativity between the subunits in the dimer; the first subunit within the dimer is cleaved much more rapidly than the second. Furthermore, since the same proportion of III is cleaved at site B among preparations with different extents of cleavage at site A, a positive cooperativity with respect to cleavage at site A may exist between subunits in the dimer if cleavage at B requires prior cleavage at A. No evidence for such positive cooperativity is seen in Figure 2, however. Although little is known about subunit cooperativity in proteolytic degradation, the facts that the susceptibility to proteolysis is greatly enhanced by polypeptide denaturation (*e.g.*, Pringle, 1970) and that denaturation is a highly cooperative process (Tanford, 1968) make such a possibility feasible.

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References

Berman, J. D. (1973), *Biochemistry 12,* 1710–1715.
Bock, E., Chen, Y. T., and Rosenberry, T. L. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* 519.
Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973), *Arch. Biochem. Biophys. 155,* 213–220.
Carpenter, F. H., and Harrington, K. T. (1972), *J. Biol. Chem. 247,* 5580–5586.
Castellano, F. J., and Barker, R. (1968), *Biochemistry 7,* 2207–2217.
Chang, H. W. (1974), *Proc. Nat. Acad. Sci. U. S. 71,* 2113–2117.
Chen, Y. T., Rosenberry, T. L., and Chang, H. W. (1974), *Arch. Biochem. Biophys. 161,* 479–487.
Cleland, W. W. (1964), *Biochemistry 3,* 480–482.
Davies, G. E., and Stark, G. R. (1970), *Proc. Nat. Acad. Sci. U. S. 66,* 651–656.
Davison, P. F. (1968), *Science 161,* 906–907.
Dudai, Y., Herzberg, M., and Silman, I. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2473–2476.
Dudai, Y., and Silman, I. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 16,* 324–328.
Dudai, Y., Silman, I., Kalderon, N., and Blumberg, S. (1972a), *Biochim. Biophys. Acta 268,* 138–157.
Dudai, Y., Silman, I., Shinitzky, M., and Blumberg, S. (1972b), *Proc. Nat. Acad. Sci. U. S. 69,* 2400–2403.
Edelman, G. M., Cunningham, B. A., Reeke, G. N., Sr., Becker, J. W., Waxdal, M. J., and Wang, J. L. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2580–2584.
Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606–2617.
Fish, W. W., Mann, K. G., and Tanford, C. (1969), *J. Biol. Chem. 244,* 4989–4994.
Froede, H. C., and Wilson, I. B. (1970), *Isr. J. Med. Sci. 6,* 179–184.
Gray, W. R. (1967), *Methods Enzymol. 2,* 139–151.
Gregory, J. D. (1955), *J. Amer. Chem. Soc. 77,* 3922–3923.
Haglund, H. (1971), *Methods Biochem. Anal. 19,* 45.
Hudson, B. G., Ohno, M., Brockway, W. J., and Castellano, F. J. (1973), *Biochemistry 12,* 1047–1053.

Kremzner, L. T., and Wilson, I. B. (1964), *Biochemistry 3,* 1902–1905.
Lee, C. Y. (1970), *Clin. Toxicol. 3,* 457–472.
Lenard, J. (1970), *Biochemistry 9,* 1129–1132.
Leuzinger, W., and Baker, A. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57,* 446–451.
Leuzinger, W., Goldberg, M., and Cauvin, E. (1969), *J. Mol. Biol. 40,* 217–225.
Mann, K. G., Fish, W. W., Cox, A. C., and Tanford, C. (1970), *Biochemistry 9,* 1348–1354.
Massoulié, J., and Rieger, F. (1969), *Eur. J. Biochem. 11,* 441–455.
Massoulié, J., Rieger, F., and Tsuji, S. (1970), *Eur. J. Biochem. 14,* 430–439.
Millar, D. B., and Grafius, M. A. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 12,* 61–64.
Powell, J. T., Bon, S., Rieger, F., and Massoulié, J. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 36,* 17–22.
Pringle, J. R. (1970), *Biochem. Biophys. Res. Commun. 39,* 46–52.
Richards, F. M., and Vithayathil (1959), *J. Biol. Chem. 234,* 1459–1465.
Rieger, F., Bon, S., and Massoulié, J. (1972a), *C. R. Acad. Sci., Ser. D 274,* 1753–1756.
Rieger, F., Bon, S., and Massoulié, J. (1973), *Eur. J. Biochem. 34,* 539–547.
Rieger, F., Tsuji, S., and Massoulié, J. (1972b), *Eur. J. Biochem. 30,* 73–80.
Rosenberry, T. L., and Bernhard, S. A. (1971), *Biochemistry 10,* 4114–4120.
Rosenberry, T. L., Chang, H. W., and Chen, Y. T. (1972), *J. Biol. Chem. 247,* 1555–1565.
Schaffer, N. K., Michel, H. O., and Bridges, A. F. (1973), *Biochemistry 12,* 2946–2950.
Seery, V. L., Fischer, E. H., and Teller, D. C. (1970), *Biochemistry 9,* 3591–3598.
Segrest, J. P., Jackson, R. L., Andrews, E. P., and Marchesi, V. T. (1971), *Biochem. Biophys. Res. Commun. 44,* 390–395.
Shapiro, A. L., Viñuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28,* 815–820.
Swank, R. T., and Munkres, K. D. (1971), *Anal. Biochem. 39,* 462–471.
Tanford, C. (1968), *Advan. Protein Chem. 23,* 121–282.
Ui, N. (1971), *Biochim. Biophys. Acta 229,* 267–581.
Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1130–1134.
Waxdal, M. J., Konigsberg, W. H., Henley, W. L., and Edelman, G. M. (1968), *Biochemistry 7,* 1959–1966.
Waxdal, M. J., Wang, J. L., Pflumm, M. N., and Edelman, G. M. (1971), *Biochemistry 10,* 3343–3347.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406–4412.
Weinberg, R. A., Loening, U., Willems, M., and Penman, S. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 1088–1095.
Williams, R. C., and Rajagopalan, T. G. (1966), *J. Biol. Chem. 241,* 4951–4954.

A Kinetic Investigation of the Interaction of Serine Transhydroxymethylase with Glycine and Serine†

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ABSTRACT: A temperature-jump kinetic study indicates five reactions occur in the serine–serine transhydroxymethylase interaction. The number of reactions observed with glycine (Cheng, C. F., and Haslam, J. L. (1972), *Biochemistry 11,* 3512) is the same; however, the glycine reactions show a 495-nm-absorbing intermediate, whereas no similar species is observed with serine. The results of a temperature and pH study are also reported for the interaction of glycine with serine transhydroxymethylase. Both the enthalpy and entropy changes for the first two steps in the reaction are positive at 25° and pH 7.3. A possible reaction mechanism for the interaction of serine and glycine with the enzyme is discussed in terms of the present experimental information.

A previous paper, Cheng and Haslam (1972), described the interaction of glycine with the enzyme, serine transhydroxymethylase. The results given in this paper describe the interaction of that enzyme with the substrate serine. The effects of temperature and pH on the reactions of glycine with the enzyme are also reported.

The spectral changes observed in the absorption spectrum of the enzyme when substrates are added facilitate both equilibrium and kinetic studies. Specifically: the spectral changes occurring with glycine or serine have been used to monitor the reactions taking place with serine transhydroxymethylase. On the basis of such changes several intermediates, a Schiff base (Metzler, 1957), *gem*-diamine (O'Leary, 1971), and a quinoid- or carbanion-type structure (Jenkins, 1964), have been postulated.

Schirch and Mason (1963) have shown that addition of glycine to a solution of the enzyme results in a decrease in the absorption at 425 nm, an increase at 343 nm, and a new peak at 495 nm. With the substrate serine, the enzyme shows no 495-nm absorption: only a slight decrease in the absorption at 343 nm, and a 4-nm shift toward shorter wavelengths in the 425-

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