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Reversible Aggregation of Acetylcholinesterase. II. Interdependence of pH and Ionic Strength*

Melba A. Grafius and David B. Millar

ABSTRACT: The reversible aggregation of acetylcholinesterase, extracted from the electroplax of *Electrophorus electricus*, was investigated in regard to the effects of pH (4.0–10.0) and ionic strength (0.1–0.5) using the sucrose gradient centrifugation technique. In ionic strengths below 0.3, at neutral pH, the enzyme reversibly aggregates into a polydisperse, rapidly sedimenting molecular form (fast components) which is in equilibrium with the slower form (slow components). In 0.1 ionic strength, at pH 5.0 and below, the fast components disappear and very rapidly sedimenting enzymatic material (pellet material) is formed and recovered in the bottom of the gradient. A study of the interaction of pH and ionic strength on the aggregation process indicates a dominant ionic effect, a delicate balance between these agents at pH 5.0, and the electrostatic nature of the regulating forces.

The complexities inherent in the observed aggregation, particularly the question of heterogeneity, are discussed. A possible participation of acetylcholinesterase, commensurate with its observed physicochemical properties, in the macromolecular complex involved in the permeability cycle of the electrogenic membrane is suggested.

The kinetic and mechanistic aspects of acetylcholinesterase (acetylcholine acyl-hydrolase, EC 3.1.1.7) extracted from the electroplax of *Electrophorus electricus* have been investigated in great detail. Consequently, today, there is a wealth of data from which some of the details of the enzymatic active site have been deduced (Wilson, 1954; Friess and McCarville, 1954; Krupka and Laidler, 1961). Information describing its macromolecular properties is, by comparison, scarce, and in some cases contradictory. For instance, the sedimentation coefficient (estimated by schlieren optics) of the enzyme has been reported to be 4 (Hargreaves *et al.*, 1963), 109 and 10.8 (Lawler, 1963), and 10.8 S (Kremzner and Wilson, 1964). Since the specific activities (in millimoles of acetylcholine hydrolyzed per milligram of protein) of the enzyme preparations used were, respectively, 66, 425, and 660, part of the discrepancies might be attributed to interference by contaminating proteins in the detection of the

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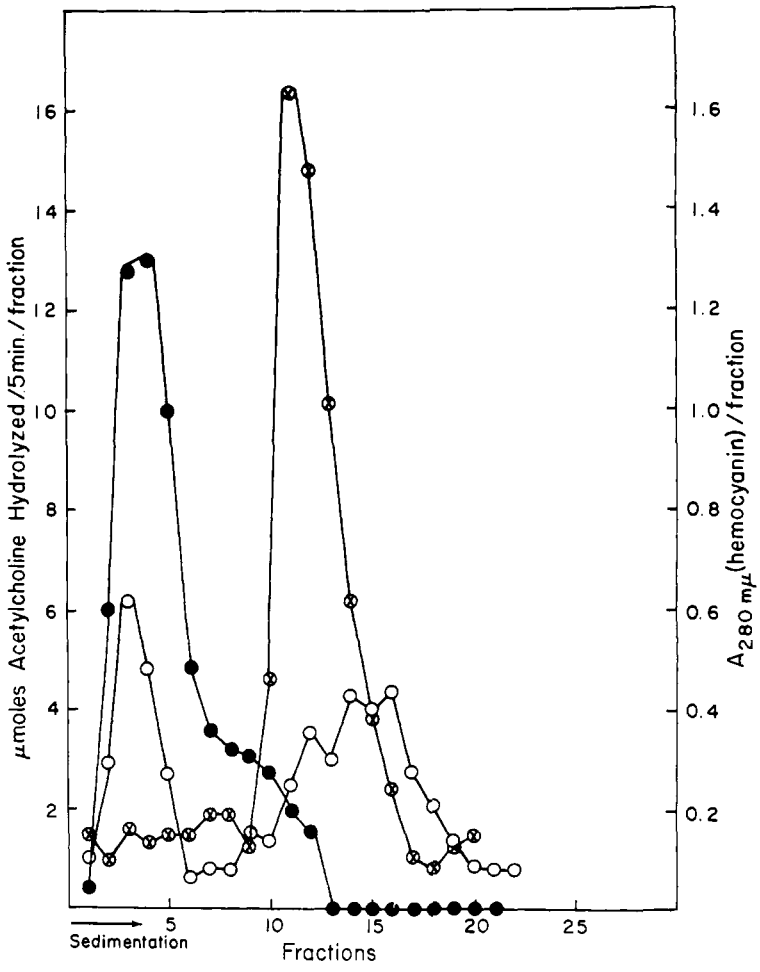

FIGURE 1: Distribution of acetylcholinesterase (AChE) activity of preparation V in sucrose gradients centrifuged at 39,000 rpm for 90 min; 15 drops/fraction. ○—○, control conditions, 0.1 M NaCl–0.015 M sodium phosphate (pH 7.1) buffer; ●—●, sample and sucrose in 1.1 M NaCl–0.015 M sodium phosphate (pH 7.1) buffer; ⊗—⊗, hemocyanin, control conditions.

moving boundary by schlieren optics. The sucrose gradient centrifugation technique in large part circumvents the problem concerning impurity since a specific feature is the ability to follow a sedimenting system by enzymatic activity. Following this approach the authors recently reported (Grafius and Millar, 1965) an ionic strength dependent, reversible aggregation between enzyme molecules sedimenting in the range of 10–14 and 65 S. The observation of a critical ionic strength region where the conversion between the major enzymatic components is sharp and rapidly reversible suggested to us that this property of the enzyme might be of significance in the permeability of electrogenic membranes where present evidence indicates the localization of acetylcholinesterase (Schlaepfer and Torack, 1966; Lewis, 1965; de Robertis *et al.*, 1963; Barrnett, 1962; Mathewson *et al.*, 1961). Accordingly, the effects of substrate and hydrolysis products on the *in vitro* molecular properties of acetylcholinesterase were investigated in an effort to observe some of the physical changes which might occur as the enzyme performed its hydrolytic role.

Methods and Materials

Purification of Acetylcholinesterase. The enzyme preparations described in this report were partially purified by the ammonium sulfate fractionation procedure of Lawler (1959). Different ammonium sulfate fractions were examined. Preparation I represented the final precipitate (20.7% $(NH_4)_2SO_4$, pH 7.9) in the third fractionation step. However, preparation II was a cut from preparation I and represented the precipitate formed in 14% ammonium sulfate at pH 4.4 in the third fractionation step; it was discarded by Lawler. In our hands, possibly because of the protein concentration (2.1 mg/ml), a considerable amount of enzymatic activity was recovered in this cut. In order to repeat Lawler's (1959) results we found it necessary at this point to reduce the protein concentration to

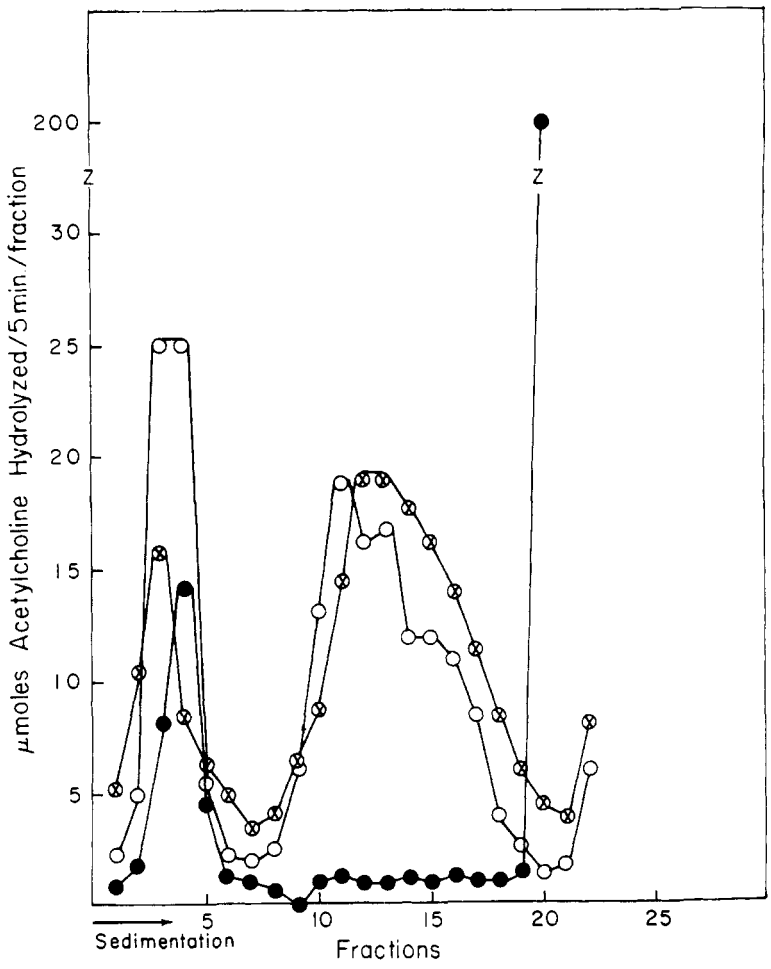

FIGURE 2: Effect of acid pH on the distribution of AChE activity of preparation II-D in sucrose gradients centrifuged at 39,000 rpm for 90 min; 15 drops/fraction. O—O, control conditions; ●—●, sample and sucrose in 0.1 M NaCl–0.015 M sodium phosphate (pH 4.0) buffer; ⊗—⊗, sample in above buffer, sucrose in control buffer.

1 mg/ml instead of the 1% solution recommended in her procedure. The above modification was employed in preparation III which was similar in other respects to preparation I, being extracted from the same batch of frozen eel tissue 6 months later. Preparation IV is the result of further purification of preparation III by high-speed centrifugation according to Lawler (1963) with the following modifications. Preparation III was dialyzed against 0.1 M NaCl–0.015 M sodium phosphate (pH 7.1) buffer for 3 days with three changes of buffer. The cloudy dialysate was centrifuged for 10 min at 20,000 rpm using the no. 40 rotor in the Spinco Model L ultracentrifuge (Spinco Division, Beckman Instrument Co.). The clear supernatant was centrifuged for 3 hr at 25,000 rpm (40,000*g*), giving a gelatinous pellet packed into the bottom of the centrifuge tube. The pellet was difficultly soluble in the above buffer and after standing 24 hr, with occasional mixing, the undissolved material was removed by centrifugation at 10,000 rpm for 10 min in rotor no. 40 in the Spinco Model L; the supernatant was designated as preparation IV. Preparation V was the fluid which could be expressed by gentle hand pressure from electroplax tissue which had been frozen for 7 months previously and which had not come in contact with any chemical agents. The storage temperature of the enzyme preparations, and the temperature at which all experimental manipulations described in this paper were executed, was approximately 4° unless otherwise stated.

Enzyme Activity. The enzyme activity, during isolation of the acetylcholinesterase preparations, was measured by an intermittent titration maintained at pH 7.4 ± 0.1 with 0.01 N NaOH, as previously described (Grafius, 1964). The reaction mixture contained 3×10^{-3} M acetylcholine chloride (Nutritional Biochemical Corp., recrystallized from hot ethanol with ether and stored over a desiccant under vacuum) and 0.0015 M sodium phosphate–0.015 M $MgCl_2$–0.1 M NaCl (pH 7.4) buffer. When indicated butrylcholine iodide (Nutritional Biochemical) was used as the substrate. Protein was determined by the absorbance difference between 215 and 225 mμ according to the method of Waddell (1956). The conversion factor used was 148 μg of protein/ΔA per ml. Specific

activity is expressed as the millimoles of acetylcholine hydrolyzed per hour per milligram of protein.

The enzyme activity in the sucrose gradient fractions was measured colorimetrically by an adaptation of Hestrin's (1949) procedure as previously described (Grafius and Millar, 1965). Substrates used were acetylcholine chloride and butrylcholine iodide, both of which had similar standard curves for the hydroxamic acid ester complexes with iron. Protein concentrations in the fractions were measured by the micromethod of Lowry *et al.* (1951).

Sucrose Gradient Centrifugation. The technique was that of Martin and Ames (1961) using 5–20% sucrose gradients. Calorimetric grade sucrose supplied by the U. S. Bureau of Standards, Washington, D. C., was used because of its low absorbance in the ultraviolet region compared with most other grades of sucrose. Enzyme sedimentation coefficients were estimated from the peak position of enzymatic activity by comparing the enzyme's movement with that of a marker of known $s_{20,w}$. For estimating the sedimentation coefficients of the *slower* components either a 1% solution of bovine serum albumin (three times recrystallized, Armour Pharmaceutical Co.), $s^0_{20,w}$ = 4.39 S (Yphantis and Waugh, 1956), or a 1% solution of catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) from bovine liver (two times recrystallized, Worthington Biochemical Corp.), $s^0_{20,w}$ = 11.4 S (Martin and Ames, 1961), were used as sedimentation markers. The catalase was assayed by the method of Beers and Sizer (1952). For the *faster* sedimenting component the marker was a 1% solution of hemocyanin prepared from the hemolymph of *Limulus polyphemus* according to the method of Felsenfeld and Printz (1959). The $s^0_{20,w}$ value, 56.7 S, measured by schlieren optics and extrapolated from data on concentrations of 1, 2, 4, and 6 mg/ml, compared excellently with a value of 56.7 reported by Eriksson-Quensel and Svedberg (1936). All markers were dissolved in the control buffer (0.1 M NaCl–0.015 M sodium phosphate, pH 7.1) unless noted otherwise. Because of the large difference in the rates of sedimentation between the fast and slow components it was necessary to determine the sedimentation coefficients for each of their respective constituents by centrifugation in separate experiments. The effective centrifugation times, $\omega^2 t$, differed by a factor of 5; the slow component's fraction could usually be resolved into several different sedimenting species by centrifugation at 30,000 rpm for 12 hr while at 39,000 rpm for 1.5 hr the fast components sedimented to the middle of the gradient, usually as a single but obviously polydisperse peak. The protein concentrations of the collected gradient fractions were measured either by absorbance at 280 mμ or by the method of Lowry *et al.* (1951).

Samples (0.1 ml of either enzyme preparation or marker solution) were layered onto the prechilled gradients and then centrifuged through the gradients for an indicated time and speed using the SW 39 rotor in the Spinco Model L ultracentrifuge. The gradients were fractionated by piercing the bottom of the cellulose tube and collecting an indicated number of drops. Control conditions will refer to gradients in which the sucrose has been dissolved in 0.1 M NaCl–0.015 M sodium phosphate (pH 7.1) buffer. A change in the NaCl concentration or pH will be noted simply in terms of the change, implying no change in the other constituents. In those experiments in which the sucrose was dissolved in buffers of pH 6 and below or pH 8 and above, the sucrose gradient fractions were neutralized immediately upon collection with 0.1 ml of 0.2 M sodium phosphate (pH 7.5) buffer. As indicated, other agents may be added to the buffer system such as acetylcholine chloride, choline chloride (Merck Chemical Co., recrystallized from hot ethanol and stored in a desiccator), sodium acetate, or ribonuclease (five times recrystallized from bovine pancreas, Sigma Chemical Co.). The pH of the buffers was adjusted with sodium hydroxide or acetic acid unless otherwise noted.

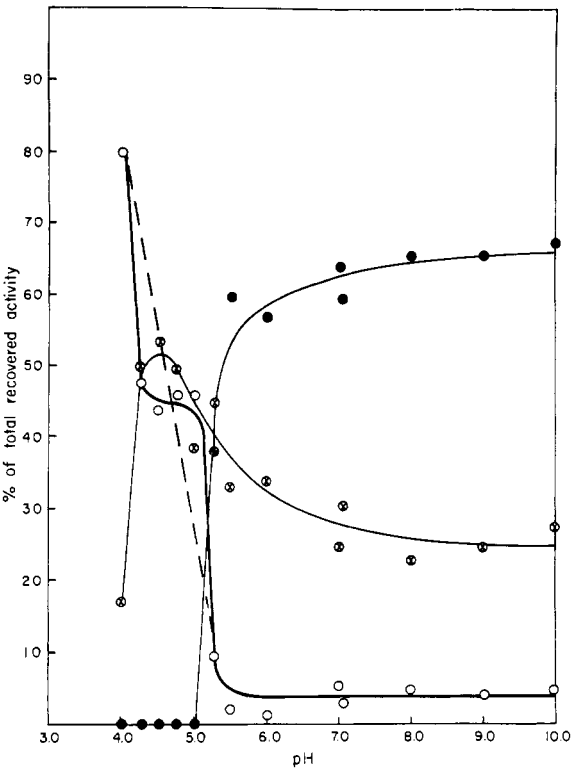

FIGURE 3: Effect of pH on the distribution of AChE activity of preparation II-D into three major sedimenting components, demonstrated in sucrose gradients centrifuged at 39,000 rpm for 90 min; 15 drops/fraction. O—O, pellet material; ●—●, fast component; ⊗—⊘, slow component.

Results

Several enzyme preparations were studied in this investigation. The method of preparation, protein concentration, specific activity, and buffer system are

TABLE I: Distribution of the Major Sedimenting Components of Acetylcholinesterase Found in Various Preparations by Centrifugation in Control Sucrose Gradients.[a]

Prepn[b]	Protein (mg/ml)	Sp Act.[c]	Enzyme Dissolved in	Slow Components (%)	Slow[f] Components (S)	Fast Components (%)	Fast Components (S)	Pellet Material[i] (%)
I	3.0	140	H_2O	58	Polydisperse[g]	—	—	21
I-D	2.5	190	Control[d]	22	9.5	41	72	24
II	3.8	30	H_2O	10	12	74	77	6
II-D	1.8	60	Control[d]	13	12	75	69	5
III	2.3	230	H_2O	64	10[h]	35	53	0
III-D	1.6	220	Control[d]	27	—	66	77	6
IV	6.5	210	Control[d]	9	—	82	77	0
V	—	—	From tissue[e]	33	—	58	69	0

[a] The sample (0.1 ml) was centrifuged for 90 min at 39,000 rpm; 15 drops/fraction unless stated otherwise. [b] See text for details of preparation. [c] Given in acetylcholine hydrolyzed per milligram of protein per hour. [d] NaCl (0.1 M)–sodium phosphate (0.015 M) (pH 7.1) buffer. [e] Fluid from electoplax applied directly to gradient. [f] Centrifuged for 12 hr at 32,000 rpm; 10 drops/fraction. [g] Five polydisperse peaks (8, 13, 14.5, 16, and 18 S), remainder of activity spread throughout gradient. [h] Lesser amounts of 12.5 and 15 S. [i] In excess of 1000 S.

shown in Table I. The pattern of distribution of enzyme activity observed in the sucrose gradient centrifugation experiments under control conditions revealed the presence of differently sedimenting components of acetylcholinesterase, as previously reported by the authors (Grafius and Millar, 1965). The approximate sedimentation coefficients are recorded in Table I as S values (S = sedimentation coefficient $\times 10^{13}$). The average value of the sedimentation coefficients for the components of a given preparation, estimated by the sucrose gradient centrifugation technique, had a precision of about $\pm 10\%$. The slow components are designated as those with S values from 8 to 18 (several are usually distinguishable in one preparation), while the fast components generally appear as one peak with S values varying from 53 to 77. In many preparations after the final ammonium sulfate precipitation it was necessary to dialyze against 0.1 M NaCl–0.015 M sodium phosphate (pH 7.1) control buffer in order for the fast component to be observed; that is, no fast component could be detected under control conditions in the sucrose gradient centrifugation experiments if the ammonium sulfate precipitate was merely dissolved in water or control buffer (see preparations I and I-D in Table I). However, in preparation II, before dialysis, 75% of the total recovered activity was present as the fast components with no change after dialysis to preparation II-D. Also preparation III contained 35% of a 53S material which changed to 66% of a 77S component after dialysis to preparation III-D. In line with the reversible, salt-dependent aggregation of acetylcholinesterase, dialysis may be necessary to remove the ammonium and/or sulfate ions which may have been more tightly bound to the enzyme molecule in some preparations than in others. Thus, it appears that preparation details, in a manner unknown at present, influence the distribution of the different molecular species of acetylcholinesterase.

The most favorable conditions for the formation or preservation of aggregates seem to be those employed in preparation II since both before and after dialysis to preparation II-D it contained a much higher proportion of fast component, 75%, than preparation I from which it was extracted (see experimental section). In preparation I no fast component was observed and after dialysis to preparation I-D only 41% of the activity was recovered as fast components in comparison to 66% in preparation III-D. It may be significant that no acid precipitate (see Materials and Methods) comparable to preparation II was removed from preparation III which also contained no fast component until after dialysis.

To gain information on the possible existence in the electroplax of the fast component we examined the fluid gently hand pressed from this tissue (preparation V). The distribution pattern of enzyme activity observed in a control gradient (Figure 1) showed a very polydisperse fast component (ranging from 60 to 80 S and accounting for 58% of the total recovered activity) as well as the usual slow components. The disaggregation of the fast components was also observed in the presence of a high ionic strength media (Figure 1). If butyrylcholine was used as the substrate in the assay, the observed enzyme activity was greatly reduced, measuring in the peak tubes no more than 0.5 unit on the activity scale of Figure 1. This indicated that very little of the activity with acetylcholine could be attributed to the nonspecific cholinesterase (acetylcholine acyl-hydrolase, EC 3.1.1.8) (Augustinsson and Nachmansohn, 1949).

TABLE II: Effect of Substrate and Hydrolysis Products on the Distribution of Acetylcholinesterase Activity in Preparation I-D Recovered from Differential Centrifugation in Sucrose Gradients.[a]

Addn to Prepn Prior to Centrifugation (M)	Addn to Control Sucrose Gradients (M)	Act. as Slow Components (%)	Act. as Fast Components (%)	Act. as Pellet Material (%)
Control	Control	27	48	23
(0.01)[b]	ACh[b] (0.01)	32	51	15
ACh[b] (0.04)	ACh[b] (0.04)	30	0	46[c]
NaAc (0.04)	NaAc (0.04)	34	40	26
HAc (0.04)	HAc (0.04)	50	0	43
HAc (0.04)	Control	32	44	22
HAc (0.04)	Ch[b] (0.04)	39	34	25

[a] The sample (0.1 ml) was centrifuged for 90 min at 39,000 rpm; 15 drops/fraction. [b] Abbreviations: ACh = acetylcholine chloride, Ch = choline chloride. [c] Remainder of activity spread evenly throughout gradient.

An investigation of the effect of substrate and reaction products on the aggregation properties of acetylcholinesterase was then undertaken (Table II). If preparation I-D was centrifuged in the presence of 0.04 M acetylcholine chloride (pH 4.2 in fractions at end of run), the more rapidly sedimenting peak (fast components) was no longer evident and the percentage of recovered total activity deposited as a pellet in the bottom of the gradient doubled. Centrifugation of the enzyme through 0.01 M acetylcholine (pH 5.2 at end of run) or 0.04 M sodium acetate (pH 7.1) revealed no major changes from the control in distribution of activity. However, if the sample was centrifuged in the presence of 0.04 M acetic acid (pH 4.2 when added to control buffer) the fast components disappeared and the amount of pellet material recovered was doubled; but the amount of slow components was also almost doubled and little tailing was observed. If acidified samples were centrifuged through either control buffer (pH 7.1) or control buffer plus 0.04 M choline chloride (pH 7.1), the amount of fast components was restored with no dramatic changes in the pattern of activity distribution, although the results with choline might be interpreted, upon comparison with the control, as a slight increase in slow components and a decrease in fast material. The similarity of the results of the acetic acid and acetylcholine experiments suggested that the disappearance of the fast components observed with acetylcholine was due to a drop in pH caused by its enzymatic hydrolysis. Indeed, the measured acidity of the fractions collected from the gradients was about pH 4.2 in both experiments.

Upon centrifugation at pH 4.0 of preparation II-D (a preparation which contained, under control conditions, a higher proportion of fast components (about 60%) than found in I-D) the fast components disappeared accompanied by the appearance of a large amount of pellet material, more than 80% (Figure 2). The reversibility of this acid effect could be demonstrated by centrifuging an acidified sample (pH 4.0) through a control sucrose gradient (Figure 2) under which conditions the fast component reappeared as 70% of the total recovered activity and the pellet material was reduced by a similar amount.

A study of the effect of pH (4.0–10.0) at a constant ionic strength of 0.1 upon the aggregation of acetylcholinesterase is summarized in Figure 3. As the pH was lowered from 10.0 the amount of slow components increased, apparently at the expense of the fast components, until pH 5.25, where the amount of fast components rapidly declined and the pellet material began to increase, although the amount of slow components was still rising. At pH 5.0 the amount of pellet material rose sharply, the fast components disappeared, the slow components increased, and a low level of activity was observed throughout the remainder of the gradient. At pH 4.75–4.25 (adjusted with HCl) a small plateau was observed in which there was little or no change in the relative contributions of slow components and pellet material, both being approximately one-half, and with no trail of activity in the remainder of the gradient. At pH 4.0 there was again a sharp increase in the amount of pellet material with a compensating decline in slow components. A significant loss in total recovered activity observed below pH 4 and above 10 dictated the limits of this experiment. It should be noted that, although at 25° the enzyme has been reported to lose activity below pH 6 and above 9 (Lawler, 1959), at 4° no change in total recovered activity was observed in this laboratory between pH 4.0 and 10.0. There may be some question about the validity of the construction of a plateau region for the pellet material between pH 4.25 and 5.0 because of the obvious scatter in the data in other regions for fast and slow components. However, the difference between the amount estimated at these pH values by means of the broken line in Figure 3 and the experimental data is too great to be due to poor precision in the technique. It is probable that a plateau area exists in this region.

A summary of the effect of ionic strength at neutral pH on the distribution of the various components

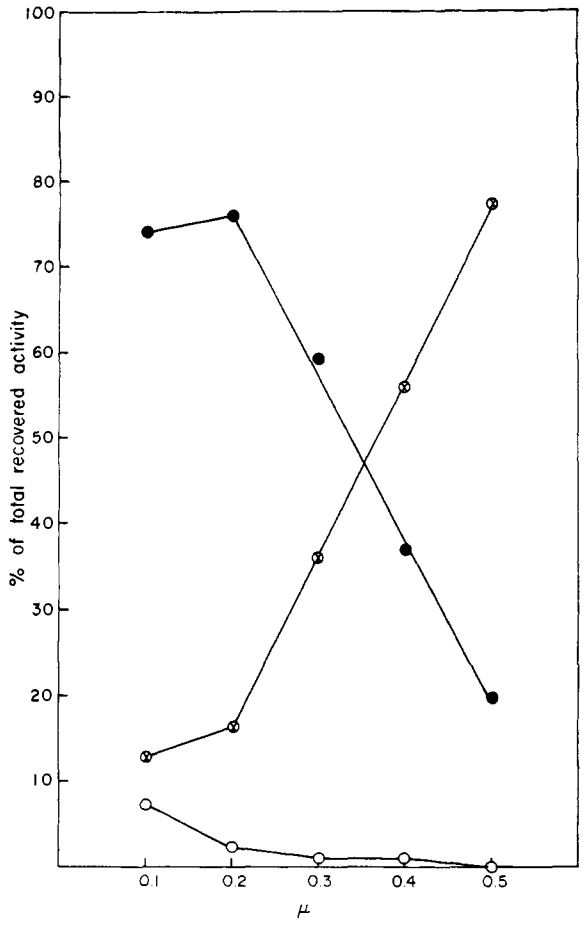

FIGURE 4: Effect of ionic strength on the distribution of AChE activity of preparation II-D into three major sedimenting components, demonstrated in sucrose gradients centrifuged at 39,000 rpm for 90 min; 15 drops/fraction. $\mu \equiv$ the molarity of NaCl in the sucrose gradient buffer containing 0.015 M sodium phosphate (pH 7.1). O—O, pellet material; ●—●, fast component; ⊗—⊗, slow component.

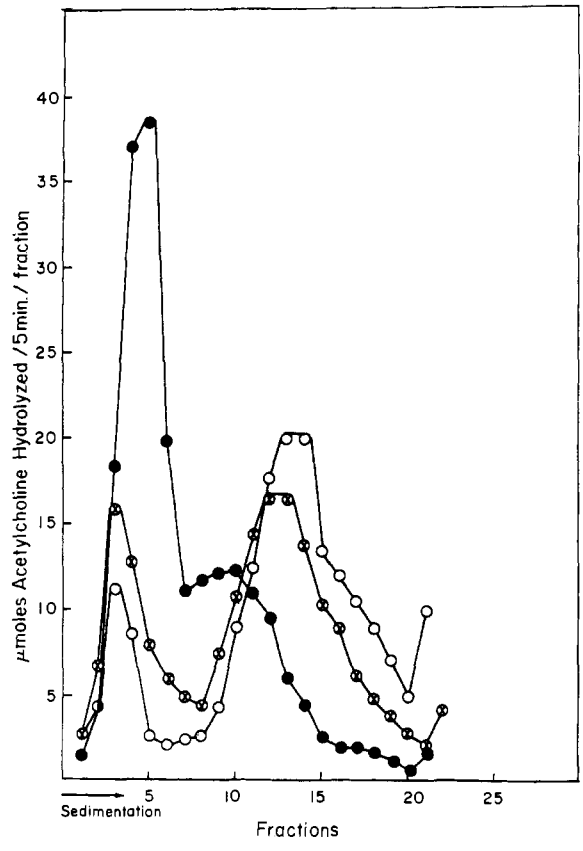

FIGURE 5: Reversible nature of the ionic strength dependent aggregation of AChE demonstrated in preparation II-D by centrifugation in sucrose gradients at 39,000 rpm for 90 min; 15 drops/fraction. O—O, control conditions; ●—●, sample and sucrose in 0.4 M NaCl–0.015 M sodium phosphate (pH 7.1) buffer; ⊗—⊗, sample in above buffer, sucrose in control buffer.

of preparation II-D is shown in Figure 4. Disaggregation to the slow components was favored at high ionic strength, particularly above 0.35 μ. The *apparent* sedimentation coefficients were also markedly affected by ionic strength. As μ increased from 0.1 the rate of sedimentation of the fast components decreased and dropped from about 70 to approximately 50 S at 0.4 μ (Figure 5). It should be noted that in the construction of Figure 4 the fast components represented the peak sedimenting faster than the slow components' peak, regardless of the S values, as long as the two could be distinguished. This decrease of the apparent sedimentation coefficient is approximately 30% and cannot be attributed alone to the nonspecific effects of increased viscosity and density due to the increased salt concentration since only an approximate 7% decrease in sedimentation coefficient would be expected by calculation from an increase of 0.1–0.4 M NaCl (Svedberg and Pedersen, 1940). A specific comformational event resulting from salt binding may be the cause or, alternately, a complex disaggregation mechanism could give rise to this result. Reversibility of the disaggregation due to high ionic strength is demonstrated in Figure 5 where a sample which was 0.4 M with respect to NaCl was centrifuged through a control sucrose gradient (0.1 M NaCl). The fast components accounted for 62% of the recovered activity in comparison with 75% found under control conditions (both sample and gradient in 0.1 M NaCl) and 37% when both sample and gradient were in a 0.4 M NaCl.

The competitive effects between low pH and high ionic strength on the distribution of the three major components is demonstrated in Figures 6 and 7. In every case the ionic effect of disaggregation dominated the pH effect of precipitation. At pH 5.0 pellet material markedly increased and no fast component was evident at 0.1 μ, but upon raising the ionic strength to 0.2 the amount of fast components was stable and no pellet material was recovered (Figure 6). However, at pH 4.0, where most of the slow components would

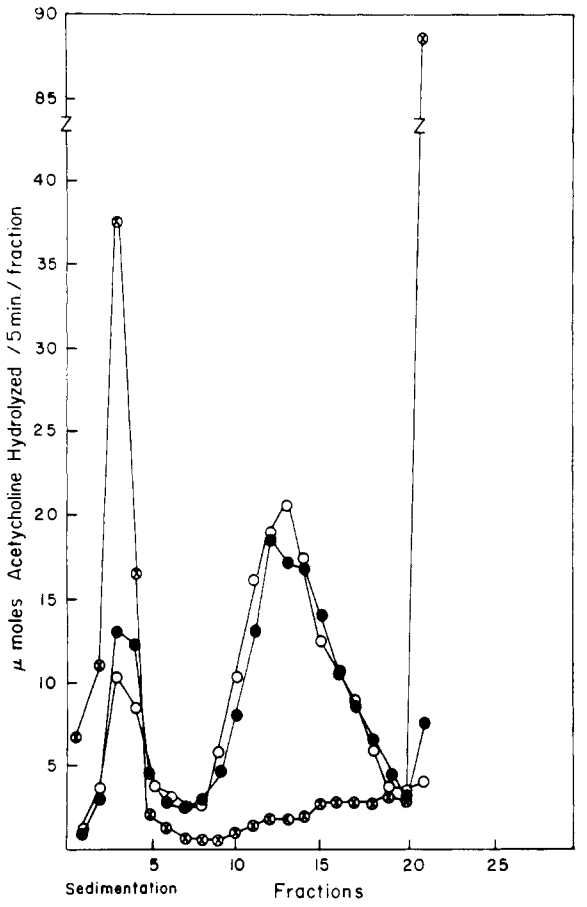

FIGURE 6: Effect of increased ionic strength on the acid-induced formation of pellet material, demonstrated in preparation II-D by sucrose gradients centrifuged at 39,000 rpm for 90 min; 15 drops/fraction. ⊗—⊗, sample and sucrose in 0.1 M NaCl–0.015 M sodium phosphate (pH 5.0) buffer; ●—●, sample and sucrose in 0.2 M NaCl–0.015 M sodium phosphate (pH 5.0) buffer; O—O, control conditions.

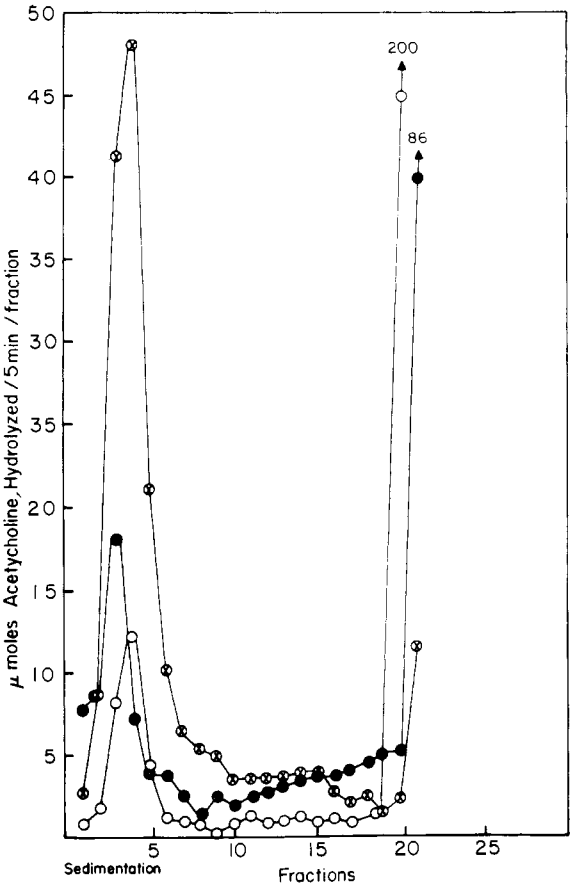

FIGURE 7: Dominant effect of ionic strength over pH in the acid-induced formation of pellet material, demonstrated in preparation II-D by sucrose gradients centrifuged at 39,000 rpm for 90 min; 15 drops/fraction. O—O, sample and sucrose in 0.1 M NaCl–0.015 M sodium phosphate (pH 4.0) buffer; ●—●, sample and sucrose in 0.3 M NaCl–0.015 M sodium phosphate (pH 4.0) buffer; ⊗—⊗, sample and sucrose in 0.5 M NaCl–0.015 M sodium phosphate (pH 4.0) buffer.

be precipitated at 0.1 μ, raising the ionic strength increased the amount of slow components and decreased the pellet material, but no fast components were evident as a discrete peak (Figure 7). It should be noted that tailing was observed in all these cases. It is not known whether the tailing is an indication of heterogeneity in size or merely of a slow rate of aggregation–disaggregation under these experimental conditions.

A gradual change in the distribution of activity for a given preparation over a period of time may be attributed to an aging factor (Grafius and Millar, 1965) in which the apparent equilibrium between the various components participating in the aggregation process shifts toward the slow components. For instance, in preparation II-D in Figure 4 at 0.1 μ the contribution of the fast components (75%) was higher than in Figure 3 at pH 7.1 (62%). The experiments in Figure 4 were performed within 1 week after the dialysis of preparation II while those in Figure 3 were about a month later. Several other experiments carried out during this month indicated a gradual increase in slow components with a concomitant decrease in the fast component. Over longer periods the enzyme preparation became cloudy, and an inactive and insoluble precipitate formed accompanied by a drop in the contribution of the fast components; after 9 months only 20% of the total recovered activity could be attributed to fast components. Because of this trend it was necessary for all comparative experiments to be completed within as short a time as possible and to be carefully controlled.

Clearly, an important question is whether the aggregation system is homogeneous or heterogeneous, *i.e.*, whether the aggregation involves only acetylcholinesterase molecules or whether the enzyme binds to other proteins or cellular constituents under proper

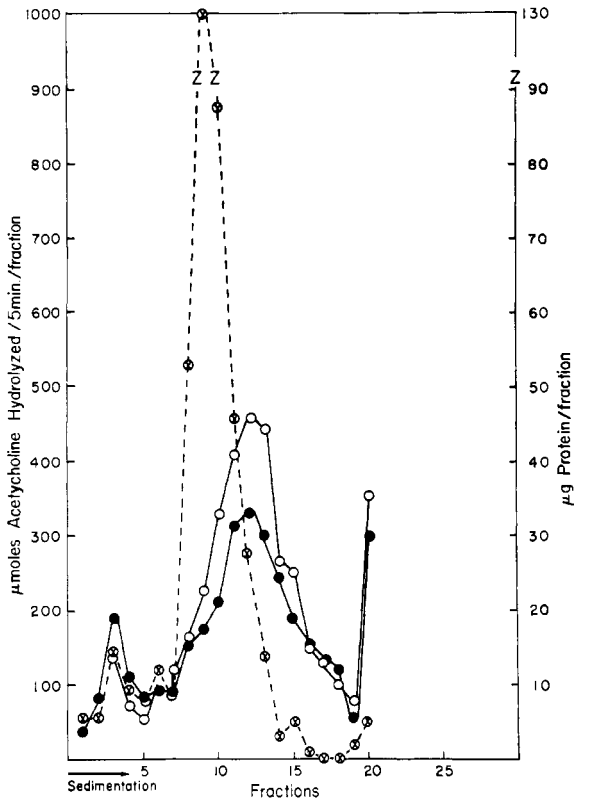

FIGURE 8: Parallel distribution of AChE activity and protein in the sedimenting species found in preparation IV, under control conditions, demonstrated by sucrose gradients centrifuged at 39,000 rpm for 90 min; 15 drops/fraction. O—O, AChE activity; ●—●, protein in O—O, copper-Folin reaction; ⊗—⊗, hemocyanin protein, copper-Folin reaction.

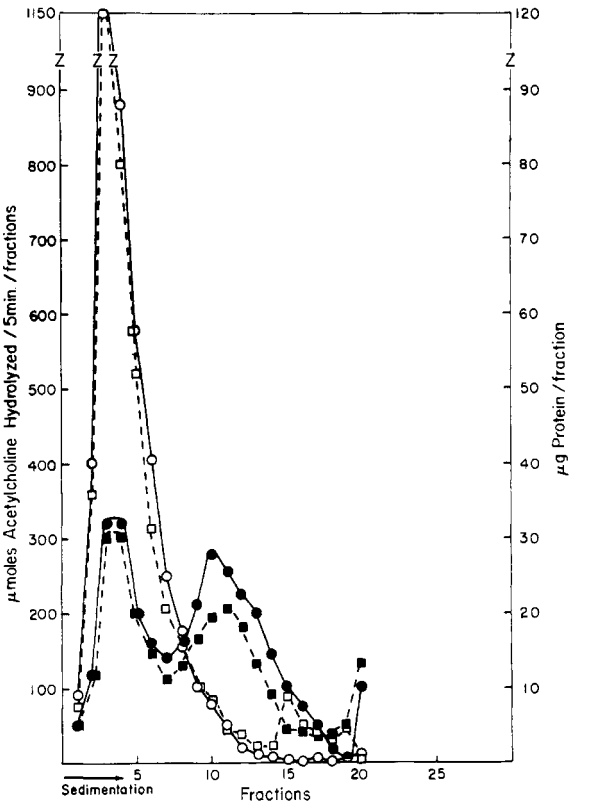

FIGURE 9: Parallel distribution of AChE activity and protein in the sedimenting species of the ionic strength dependent, reversible aggregation found in preparation IV, demonstrated by sucrose gradients centrifuged at 39,000 rpm for 90 min; 15 drops/fraction. O—O, AChE activity, sample and sucrose in 0.8 M NaCl-0.015 M sodium phosphate (pH 7.1) buffer; □—□, protein in O—O, copper-Folin reaction; ●—●, AChE activity (sample in above buffer, sucrose in control buffer); □—□, protein in ●—●, copper-Folin reaction.

conditions of ionic strength and pH. Thus, protein and enzymatic activity distibutions under conditions of high ionic strength were compared in the more highly purified preparation IV. Under control conditions activity and protein zones were parallel (Figure 8). If the fast components (82% of 77 S under control conditions) were disaggregated by 0.8 M NaCl (Figure 9) and then reaggregated by centrifugation into 0.1 M NaCl (Figure 9) the reversibility was not complete (51% of 58 S) but the patterns of enzyme activity and protein distribution were almost identical. The same parallel relationship between protein and enzymatic activity among the slow components formed by disaggregation in 0.8 M NaCl is shown in Figure 10 where the increased effective centrifugation time permitted the slow components to sediment to the middle of the gradient.

Since RNA itself participates in an aggregation process and since it is an integral part of nearly all tissue its possible participation in the acetylcholinesterase-aggregating system was considered. However, this was ruled out in an experiment where preincubation of 60 µl of preparation II with 50 µl of a 3-mg/ml solution of ribonuclease in control buffer for 1 hr at 25° did not affect the sedimentation rate of the fast component. Observation with schlieren optics of the change of the sedimenting peak, approximately 5 S, of polyuridylic acid (Miles Laboratories, Inc.) to a polydisperse, low molecular weight distribution after incubation with the ribonuclease verified the activity of the enzyme.

The effect of temperature upon the aggregation process and, particularly, upon the $s_{20,w}$ values for the components estimated from the sucrose gradient centrifugations appeared to be negligible in several experiments in which preparation II-D was centrifuged through a gradient at approximately 27°, *i.e.*, the gradient was at room temperature before applying the sample and the temperature of the chamber at the end of the run was 30°. Note that sedimentation coefficients estimated by the sucrose gradient method are automatically corrected to 20° when using the $s^0_{20,w}$ for the marker The fast component was somewhat

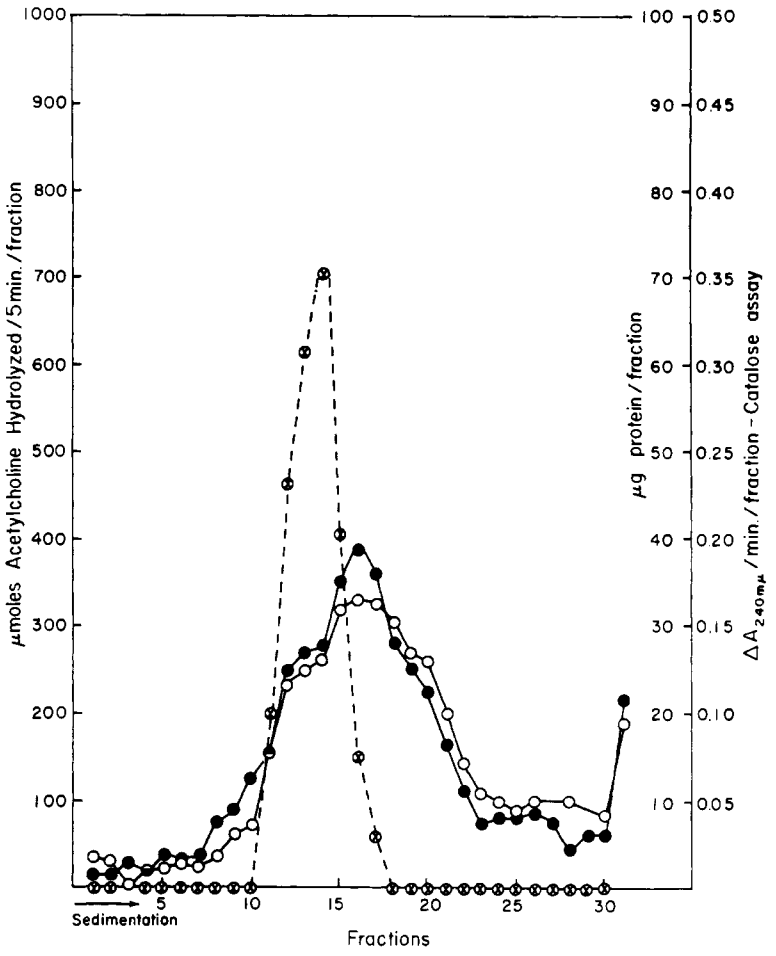

FIGURE 10: Parallel distribution of AChE activity and protein in the slow components of preparation IV in the presence of high ionic strength, demonstrated by sucrose gradients centrifuged at 28,000 rpm for 16 hr; 10 drops/fraction. o—o, AChE activity (sample and sucrose in 0.8 M NaCl–0.015 M sodium phosphate (pH 7.1) buffer); ●—●, protein in o—o, copper–Folin reaction; ⊗—⊗, catalase activity (sample and sucrose in 0.01 M Tris (pH 7.5) buffer).

more diffuse but the peak fraction corresponded to an S value of 75, in agreement with the value obtained under the standard conditions of 4°. A sharp meniscus between the sample and gradient could not be achieved if the sample was at room temperature when applied to a gradient at room temperature, resulting in a very diffuse distribution pattern which could not be evaluated. The sedimentation coefficients of the fast components estimated by sucrose gradient centrifugations in preparation IV were shown not to be dependent upon concentration when diluted 100-fold with control buffer from 6.5 to 0.065 mg/ml; S values were 77 and 76, respectively.

Discussion

The question of the molecular size of acetylcholinesterase extracted from the electric organ of *E. electricus* deserves careful consideration. The discrepancy in sedimentation coefficients ($s^0_{20,w}$) between the polymer reported by Lawler (1963), 94–109 S, and the fast components under consideration in this paper, 69–77 S, is still not understood. We have assumed that sucrose does not specifically bind to the enzyme, thus altering the partial specific volume or frictional ratio. It may be noted that the $\bar{V}$'s of the enzyme, 0.750 (Lawler, 1963), and hemocyanin, 0.735 (Svedberg and Heyroth, 1929), are close and thus do not cause an error of sufficient magnitude to explain the difference between Lawler's results and ours. A dilution factor obviously exists between centrifugation conditions in sucrose gradients and the classical moving-boundary methods. However, within limits of the gradient technique, concentration effects appear to be negligible since a 100-fold dilution did not change the rate of sedimentation of the fast component (Grafius, 1964). Also, a temperature effect is probably not a contributing factor nor does purity appear to be, at least in the range of specific activities investigated here (Table I). It may be that a difference in some unidentified experimental parameters results in aggregates with different apparent average molecular sizes. Certainly, the

distribution and value of sedimentation coefficients of the fast and slow components appear to vary from preparation to preparation (Table I) (Grafius and Millar, 1965). Moreover, in some preparations dialysis appears to make a difference in the distribution and value of sedimentation coefficients; in others no effect is noted. However, the sedimentation coefficient distribution of the slow component fraction does not seem to determine the value of the fast component (Grafius and Millar, 1965). Experimental conditions did differ between the two laboratories in respect to Mg^{2+} which was present as 0.015 M in Lawler's (1963) preparation during dialysis and in the pelleting procedure but not in ours. We have previously shown that this small amount of Mg^{2+} in the dialysis buffer affected neither the quantity nor the S value of the fast components in our earlier preparations (Grafius and Millar, 1965) but there may be subtle reasons for an effect of Mg^{2+} on the enzyme reflected by the sedimentation coefficient of the aggregates formed during the pelleting procedure.

Dialysis sometimes increases the specific activity of a preparation (Table I), but this cannot be taken as an indication of the increase in specific activity for the fast components *per se*. Note that specific activity may increase without an increase in fast component (II → II-D) and that an increase in fast component need not be accompanied by an increase in specific activity (III → III-D). Indeed, loss of small ultraviolet-absorbing molecules through the dialysis sac could account for the apparent increase in specific activity. It should be noted that the assay media is always approximately 0.1 μ and could mask changes in activity owing to size, particularly if the aggregation–disaggregation process is rapidly reversible. Changeaux (1966b) has also discussed the difficulty in relating activity changes to salt-induced changes in the degree of aggregation. However, the kinetic properties of the slow and fast components may differ in other ways. In this connection an examination of the data presented by Wilson and Cabib (1954) and Friess *et al.* (1954), both of whom were concerned with the essentiality of Mg^{2+} for the enzymatic activity of acetylcholinesterase, indicates that the differences reported by them could possibly be interpreted as a difference in Mg^{2+} requirements for slow and fast components, for the preparations of Wilson and Cabib (1954) were stored in a low ionic strength buffer (I. B. Wilson, personal communication), and in this case exogenous Mg^{2+} apparently was potentiating but not essential, while in the preparations of Friess *et al.* (1954) the ionic strength was 0.8 (5% ammonium sulfate), and here exogenous Mg^{2+} was essential. However, if the preparations of Friess were diluted to 0.03 μ a Mg^{2+} requirement similar to that reported by Wilson and Cabib could be observed only after 24 hr, suggesting a possible slow dissociation of strongly bound NH_4^+ and/or SO_4^{2-} and subsequent aggregation to fast component.

Kremzner and Wilson (1964) found no evidence for polydispersity in their preparations, which were very highly purified by chromatographic procedures, raising the question that polymeric forms may be artifacts of preparation. However, we observed the fast component in the fluid which flows, under gentle pressure, from the electric organ tissue (Figure 1) and in the final toluene exudate (Grafius and Millar, 1965) which is an early stage in the purification of the enzyme, suggesting that part of the acetylcholinesterase in the tissue may exist in an aggregated form. Lawler (1963) has also speculated on this possibility. Recently, using the sucrose gradient centrifugation technique, Changeaux (1966a) has reported on an aggregated form of acetylcholinesterase found in impure preparations extracted from the electric organ of the *Torpedo marmorata*. The aggregated, highly polydisperse form had an apparent peak (50 S) that could be disaggregated by 0.1 M $MgCl_2$ or 0.3 M KCl to a slower component (14 S). But the question of a homogeneous or heterogeneous aggregation remains moot because of the high opportunity for association with other proteins and other cellular constituents in the process of enzyme extraction. Although a nearly parallel distribution between activity and protein concentration (Figures 8–10) may be observed, this cannot be solely interpreted as evidence for a homogeneous aggregation since the specific activity of this preparation was only 220. Perhaps the enzyme is associated with another protein having the particular sedimenting and aggregating properties observed here.

Participation of other cellular constituents must be considered. For instance, several small, biologically important molecules have been shown to be an essential part of the *in vitro* conditions which will induce a change in protein conformation and/or size of certain enzymes; *e.g.*, DPN^+–DPNH[1] and ADP–ATP on glutamic dehydrogenase (Tomkins *et al.*, 1963; Bitensky *et al.*, 1965), ATP on deoxythymidine kinase (Bresnick *et al.*, 1966), and TPN^+ on glucose 6-phosphate dehydrogenase (Kirkman and Hendrickson, 1962). Also, electric organ tissue is rich in mucopolysaccharides (Hassón and Chagas, 1961), which may suggest binding with sugars as another possible contributing factor and, in fact, it has been reported that much of the acetylcholinesterase from the electric organ of *T. marmorata* appeared to be electrophoretically inseparable from mucopolysaccharides (Toschi and Marini-Bettòllo, 1956). Because of the ubiquitous distribution of ribonucleic acid, its association with the enzyme in nerve tissue was given serious consideration. However, this possibility was ruled out in an experiment in which incubation with ribonuclease did not affect the distribution of the fast- and slow-sedimenting species of the enzyme. As a matter of course, butyrylcholinesterase, always a possible contaminant in acetylcholinesterase preparations, was eliminated by assay with its specific substrate, butyrylcholine (Augustinsson

[1] Abbreviations used: DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotides; ADP and ATP, adenosine di- and triphosphates; TPN, triphosphopyridine nucleotide.

and Nachmansohn, 1949), with negative results.

An effect of the enzyme substrate, acetylcholine, could not be observed in the sucrose gradient centrifugation experiments since the hydrolysis product, acetic acid, had a pronounced effect of its own which was shown largely to be an effect of pH and not of acetate or choline ions *per se* (Table II). The different pH values at which fast and slow components may apparently be converted to pellet material (Figure 3) can be attributed to electrostatic forces which differ in some way between the two predominant, average molecular sizes, *i.e.*, the fast and slow components. A particularly discriminating balance between pH and ionic strength was observed at pH 5.0 where the fast components disappeared if the ionic strength was 0.1 but remained if the ionic strength was raised to 0.2 (Figure 6). This effect may be due to a salt-induced change in the pK's of the groups presumably involved. However, at pH 4.0 an increase in ionic strength did not protect the fast components (Figure 7) but rather increased the ratio of slow components to pellet material accompanied by a heterogeneous spread of sedimentation coefficients. These observations, together with the apparent dissociation of fast components to slow components as the pH decreased from 10 to 5 and the continued increase of slow components after the fast component had disappeared at this pH (Figure 3), suggest that pellet formation may be the result of a process involving dissociation of fast components to slow components and subsequent reaggregation.

It is difficult not to speculate upon the possibility of a role for acetylcholinesterase as a part of the system which regulates the permeability of electrogenic membranes, one that is distinct from its enzymatic function as a scavenger for acetylcholine and is dependent upon the localized fluctuations of ionic strength and pH which occur during excitation. There is some evidence in the literature which indicates that acetylcholinesterase is an integral part of the ultrastructure in several different types of excitable membranes including certain axonal (Schlaepfer and Torack, 1966; Lewis, 1965), junctional (Lewis, 1965; de Robertis *et al.*, 1963; Barrnett, 1962; Mathewson *et al.*, 1961), and muscle membranes (Barrnett, 1962). The enzyme has also been reported to be in the vesicular structures found in the terminal axoplasm at the myoneural junction (Barrnett, 1962). It has been suggested by Green (Green, 1965; Green and Perdue, 1966) that all membranes may be composed of molecular repeating units which are made up of specific associated elements of catalytic and structural proteins and phospholipids arranged in a modular form favorable to the integrated metabolic sequences peculiar to a given membrane. He has assembled much evidence which is certainly consistent with such a hypothesis for the mitochondrial membrane. Using this line of reasoning in electrogenic membranes, acetylcholinesterase could be a part of a postulated base piece and essential to the continuum of the membrane. Then, under a proper interplay of ionic strength and pH, the continuum would be fleetingly interrupted to permit passage of cations across the concentration gradients found in resting membranes. Certainly the abundance of charged groups (proteins and phospholipids) found in membranes makes it reasonable to speculate upon the effects of pH in the permeability cycle. Indeed such a postulate can be seen to be compatible with previous theories concerning the permeability phenomenon (Meyer, 1937; Bergmann and Shimoni, 1952; Tasaki, 1963; Tasaki and Singer, 1965). Acetylcholinesterase itself has been suggested as the long-sought receptor protein (Župančič, 1953; Changeaux, 1966b) which supposedly increases the permeability of certain electrogenic membranes upon binding with acetylcholine, according to two different mechanisms postulated for the conduction and transmission of nerve impulses; one proposes a neurohumoral secretion of acetylcholine and is limited to transmission at synaptic junctions (Fatt and Katz, 1951; Castillo and Katz, 1956; Katz, 1962; Eccles, 1964) while the other is a unified theory of chemical control for all electrogenic membranes and requires an intracellular release of acetylcholine (Nachmansohn, 1946, 1955, 1959, 1966).

With the above considerations in mind, it is suggested that the salt- and pH-dependent reversible aggregation of acetylcholinesterase might play a role in the control of the permeability cycle operating in electrogenic membranes, but it is also stressed that this enzyme may not be a unique protein in such a system but that other proteins and cellular constituents could be involved in this admittedly hypothetical membrane. It is further emphasized that the aggregates observed *in vitro* may not actually exist as such in the membrane, but that merely the observed physicochemical properties of the enzyme are commensurate with a role in the permeability cycle of excitable membranes.

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