MULTIPLE MOLECULAR FORMS OF CHOLINESTERASE FROM ELONGATED ANIMALS

Thesis by

Carl Douglas Johnson

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Abstract

Cholinesterase activity in Electrophorus electricus and Caenorhabditis elegans has been fractionated, primarily by velocity sedimentation in sucrose gradients. The electric organs of E. electricus contained five separable forms (7.5 S, 9 S, 12 S, 14 S and 18 S). Three, so-called native forms (9 S, 14 S and 18 S) are insoluble in low-ionic strength solutions and they were all shifted to higher sedimentation constants by treatment with bacterial collagenase. The other two (globular) forms are soluble in low-ionic strength solutions and unaffected by collagenase. Trypsin converts the native forms to the 12 S form. Neither collagenase nor trypsin affect the molecular size of the active subunit as determined by SDS-polyacrylamide gel electrophoresis.

Four forms of cholinesterase activity were separated from extracts of the soil nematode Caenorhabditis elegans (5 S, 7 S, 11 S, 13 S). The smaller two forms as a pair and the larger two forms as a second pair are kinetically similar (Kms, substrate and inhibitor specificity). There are significant differences between the pairs. A screening procedure using the selective inactivation of the 5 S and 7 S forms by the anionic detergent sodium deoxycholate has been applied to 89 mutants of C. elegans. One uncoordinated mutant (BC46) apparently devoid of 11 S or 13 S activity was identified. The 5 S and 7 S

forms in this mutant are unaltered. The behavioral defect is limited to the body region. Head movements and sensory responses to mechanical, chemical and osmotic stimuli seem unaltered. The mutation is X-linked.

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General Introduction

The suggestion that organisms have enzymes capable of hydrolyzing the ester linkage of acetylcholine is as old as the chemical identification of ACh as a pharmacologically active substance (Dale, 1914). After it had been demonstrated that acetylcholine was a neurotransmitter, it was assumed that *in vivo* these cholinesterases functioned to terminate the action of the transmitter and this assumption received strong experimental support from biochemical studies showing that the enzyme was concentrated at the synapses of cholinergic motoneurons in vertebrate skeletal muscle (Marnay & Nachmansohn, 1938). Further and more comprehensive studies of the localization of cholinesterase activity has since revealed anomalous localizations in structures containing no cholinergic synapses (e.g. in erythrocytes and plasma). Furthermore, biochemical studies of single neurons in lobster (Hildebrand, Townsel, & Kravitz, 1974) and in *Aplysia* (Giller & Schwartz (1971) have demonstrated little difference in the level of cholinesterase between cholinergic cells and cells using other transmitters. Although a number of functions have been suggested for non-synaptic cholinesterase (e.g. membrane permeability, Duncan, 1967; axonal conduction, Nachmansohn, 1946) none of the suggestions have received much experimental support and most have been discredited by clear demonstrations

of the presumed cholinesterase-dependent function in the absence of the enzyme. Two observations suggest that the function of some cholinesterases may not be essential; 1) comparative studies often show large differences in the levels of cholinesterase activity among closely related organisms (Hart & Lee, 1966; Zagicek, 1957) and, 2) genetic variants have been identified in which particular cholinesterase activities are eliminated without producing a recognizable phenotype (Rubenstein, et al., 1970).

In many organisms cholinesterases occur in several separable molecular forms. In studying the properties of these forms, one can hope to learn whether they result from the expression of multiple genes or from the formation of associations between a common active subunit and other components. Forms with a common active subunit may represent sequential stages in the formation of complexes with specific localizations and functions, they may be involved in transport of the enzyme, or they may result from degradative processes. If specific forms could be shown to be localized at synapses, the appearance of these forms could provide a biochemical marker for the events of synapse formation.

The studies presented in this thesis concern the multiple forms of cholinesterase activity in two elongated

organisms: the electric eel Electrophorus electricus and the soil nematode Caenorhabditis elegans. Part I describes a rapid, radiometric liquid extraction assay for cholinesterase activity without which the large number of assays involved in fractionating and characterizing separable forms would not have been possible.

Acetylcholinesterase has been directly solubilized from the electric organs of E. electricus with buffered 1 M NaCl (Silman & Karlin, 1967) and fractionated by velocity sedimentation into three "native" forms (9 S, 14 S and 18 S (Massoulie & Rieger, 1969). These forms are insoluble in low-ionic strength solutions. Two additional "degraded" forms (7.5 S and 12 S) are found in autolyzed preparations. These forms can be produced by proteolytic treatment or by sonication of the native forms (Massoulie, Rieger & Tsuji, 1970; Massoulie, Rieger & Bon, 1971). They are soluble in low ionic strength solutions. The kinetic properties of the forms do not differentiate them (Massoulie & Rieger, 1969) and antibodies prepared against any form will precipitate all of the forms (Rieger, et al., 1973b; Gurari, Silman & Fuchs, 1974). These characteristics suggest that all of the forms have the same active subunit. The differences between the "native" and the "degraded" forms lies in the association of the active subunits with an elongated

(500 Å) tail which has been observed in the electron microscope (Rieger et al., 1973a). In part II, a solubility based fractionation procedure was used to facilitate the separation of all five forms. Treatment with bacterial collagenase selectively shifted the three native forms to faster sedimenting forms. This suggests that the native forms may contain a collagen-like component, presumably in the tail.

Caenorhabditis elegans is a small (~1 mm long) free living nematode whose self-fertilizing hermaphroditic mode of reproduction greatly facilitates the isolation of recessive mutants (Brenner, 1974). The nervous system of the animal contains only about 300 cells. Parts III and IV concern the separation and characterization of four separable forms of cholinesterase activity in wild type C. elegans and in an uncoordinated mutant in which two of these forms are missing.

The function of cholinesterases in nematodes is unknown. Electrophysiological studies in the large nematode Ascaris lumbricoides have shown 1) that bath application of 5×10^{-6} M ACh produces a measurable depolarization of the muscle cell membrane and 2) focal application of ACh is effective only when directed towards the region of neuromuscular junctions suggesting that ACh is a transmitter at neuromuscular synapses

(del Castillo, de Mello & Morales, 1963). However, the complex anatomy of this region (the muscle cells send processes which approach the nerve cord and form a complex synctium under which the neuromuscular synapses occur (Rosenbluth, 1965)) prevents the study or reproduction with iontophoretic application of the actual release of transmitter which is usually required to firmly establish a particular case of chemical transmission. It has also not proved possible to determine which cells release the transmitter. Histochemical staining for cholinesterase has been reported in Ascaris muscle (Lee, 1962).

Biochemical studies of nematode cholinesterases have largely concerned themselves with the mode of action of anthelminthics(Hart & Lee, 1966; Knowles & Casida, 1966). One result of these studies has been to measure levels of cholinesterase activity in a variety of parasitic nematodes (Hart & Lee, 1966). What is most striking is the variation (from 0.3 u/gm to 300 u/gm).

Cholinesterase from the nematode Nippostrongylus brasiliensis (a rat intestinal parasite) has been fractionated by gel electrophoresis into three bands, two fast and one slow (Edwards, et al., 1971). The proportions of these forms are altered in nematodes who survive the host's immune responses (Jones & Ogilvie, 1971). Furthermore rats infected with this parasite acquire

antibodies to nematode cholinesterases. In some cases, these antibodies react only with the two fast bands, in others they react with all three bands. The level of activity observed in the slow band seems to be related to its antibody. If non-immune rats are given an antibodies from an immune rat, the level of the slow band of nematode cholinesterase rises. Alternatively, if immune rats are suppressed by injection of cortisone, the level of the slow band falls. Lee has shown that cholinesterase in this animal is localized in the anterior glands (Lee, 1970).

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I A Rapid, Simple Radiometric Assay for Cholinesterase, Suitable for Multiple Determinations

CARL D. JOHNSON AND RICHARD L. RUSSELL

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A Rapid, Simple Radiometric Assay for Cholinesterase, Suitable for Multiple Determinations[1]

CARL D. JOHNSON AND RICHARD L. RUSSELL

Division of Biology, California Institute of Technology, Pasadena, California 91109

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A rapid and simple radiometric assay for cholinesterase, suitable for multiple determinations, has been developed. (^{3}H-acetyl) choline is enzymatically hydrolyzed in a small reaction volume in a scintillation vial. The released [^{3}H]acetate is then extracted into a toluene-based scintillator added directly to the vial, without removing the reaction volume. The extracted [^{3}H]acetate counts efficiently, but the unhydrolyzed [^{3}H]acetylcholine remains unextracted in the small aqueous reaction volume, from which its weak β-particles of decay do not escape to excite the scintillator. The assay is highly reproducible, quite sensitive, and useful for applications in which multiple samples must be quickly assayed.

Many techniques for the quantitative assay of cholinesterase have been described and recently reviewed (1). Among these, radioisotopic assays based upon the estimation of [^{3}H] or [^{14}C]acetate produced by hydrolysis of labeled [^{3}H] or [^{14}C]acetylcholine are particularly valuable for their sensitivity and their usefulness over a wide range of substrate and enzyme concentrations.

The available radioisotopic assays for cholinesterase differ, in essence, only in the procedure used to separate the labeled acetate product from unhydrolyzed labeled acetylcholine substrate. Winteringham and Disney (2) measured the acetylcholine remaining following acidification and drying of assay samples to remove "volatilized" (i.e., protonated) acetate. Reed *et al.* (3) bound acetylcholine with an ion-exchange resin, centrifuged and measured labeled acetate in an aliquot of the supernatant. Potter (4) selectively extracted acetate from acidified reaction mixtures into an organic solvent system consisting of 5:1 toluene:isoamyl alcohol. McCaman *et al.* (5) selectively removed labeled acetylcholine by precipitation as the reinecke salt in 0.33 M HCl, and Wilson *et al.* (6) removed it by passage over a cation exchange column.

In each of these techniques, the sample must be taken through at least

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one postassay manipulation (usually centrifugation or passage over a column) before counting; consequently these techniques are not convenient for assaying the large numbers of samples which are usually encountered in fractionation schemes to purify cholinesterase or to analyze its molecular properties. In this paper we describe a radioisotopic assay which is convenient for these purposes because it keeps postassay manipulations to an absolute minimum.

The assay is based in principle on the liquid-extraction technique of Potter (4), but includes a few simple and important modifications. [^{3}H]acetyl-labeled acetylcholine is used as substrate for hydrolysis in a reaction carried out in a scintillation counting vial. After hydrolysis, the reaction mixture is stopped by addition of strong pH 2.5 buffer; this protonates the ^{3}H-labeled acetate produced, and allows it to be efficiently extracted into a large volume excess of toluene-based scintillation fluid added directly to the scintillation vial. Without further additions, the vial is simply counted; the ^{3}H-labeled acetate in the organic phase is counted efficiently, but the ^{3}H-labeled acetylcholine which remains in the small volume of aqueous reaction mixture does not count because the weak β particles from the ^{3}H decay are trapped in the aqueous phase before reaching the scintillator. In short, then, the assay selectively measures enzymatically produced [^{3}H]acetate by using two simple fixed-volume additions to a subsequently counted scintillation vial.

MATERIALS AND METHODS

Acetylcholinesterase of the electric eel *Electrophorus electricus* was the ECHP preparation from Worthington Biochemical Corp., or the preparation 461-2 from P-L Biochemicals. [^{3}H]acetylcholine chloride was the preparation TRA-277, 290 mCi/mmole from Amersham-Searle. [^{3}H]sodium acetate was the preparation TRA-12, 500 mCi/mmole from Amersham-Searle. All other chemicals were standard reagent grades.

Labeled Acetylcholine Substrate

One millicurie of (^{3}H-acetyl)choline chloride was dissolved in 2.0 ml H_2O (measured pH 4–5, presumably by equilibration with atmospheric CO_2) and stored frozen at −20°C. As received, samples contained 2–5% [^{3}H]acetate. For most purposes this level of [^{3}H]acetate could be tolerated as background in the assay, permitting use of the substrate without further purification. When required, the background was reduced to 0.2% by; (1) addition of 0.005 ml glacial acetic acid/ml of [^{3}H]acetylcholine; (2) repeated extraction of [^{3}H]acetate with 10 vol of toluene:isoamyl alcohol (10:1); (3) repeated extraction with 100% toluene to remove isoamyl alcohol from the aqueous phase; and (4) blowing with an air stream to remove residual toluene. The 0.2%

background level achieved in this way was not stable, rising to 2–5% upon storage at −20°C over a period of a few weeks. Although *in vacuo* storage of [^{3}H]acetylcholine to provide continuous removal of [^{3}H]acetate was envisaged as a method for stabilizing the low background level, it was not tried.

The [^{3}H]acetylcholine chloride solution was diluted with freshly prepared solutions of unlabeled acetylcholine iodide to provide labeled substrate at appropriate concentrations and specific activities. Assays were routinely performed at two acetylcholine concentrations—2×10^{-3} M and 5×10^{-5} M. Substrate was prepared at 5× these concentrations (i.e., 10^{-2} M and 2.5×10^{-4} M), at specific activities of 1 mCi/mmole and 40 mCi/mmole, respectively, so that 0.020 ml would in either case yield about 10^5 cpm upon complete hydrolysis. (Under the standard assay conditions, 70–75% of the [^{3}H]acetate produced by hydrolysis was extractable and countable, and our counting efficiency for extracted [^{3}H]acetate was 45–46%.) Aliquots of substrate sufficient for about 200 assays were prepared and stored at 4°C; under those conditions the background level of extractable label was quite stable for several weeks at 2–5%.

Standard Assay Procedure

The standard assay mixture contained 0.05 ml of 0.05 M potassium phosphate buffer (pH 7.0), 0.02 ml of acetylcholine substrate at the required concentration (see above), and enzyme plus H_2O to give a final volume of 0.1 ml. When equal aliquots of many fractions were to be assayed, the buffer, substrate, and water were premixed and aliquoted to the reaction vessels, and reactions were started shortly thereafter by addition of enzyme from the fractions. The reaction vessels were small (1 dram) glass vials (cat. no. 7475, Rochester Scientific Co., Inc., purchased with plastic caps) suitable for later insertion into standard widemouth scintillation vials. Incubations were carried out at room temperature (25 ± 1°C) except where indicated otherwise. After a suitable incubation time (10 min was frequently adequate), the reaction was stopped by adding 0.1 ml of stopping mixture (see below), followed immediately by 4.0 ml of scintillation mixture (see below) and the vials were capped, shaken, and inserted into wide-mouth scintillation vials for counting. The entire stopping procedure could be accomplished, with a little practice, in 12–15 sec, using automatic pipettors for the stopping and scintillation mixtures, so that a large number of assays could be run simultaneously by using staggered starting and stopping times. When the occasion demanded, we were able to perform up to 300 assays in a total time of 2.5 hr. Counting of the vials (in a Beckman LS-200 scintillation counter) was usually initiated 30 min after stopping the reaction, since

with this precaution a single vigorous shaking of the vials at the time of stopping provided a complete and stable extraction of acetate and good separation of aqueous and organic phases.

Stopping Mixture

The purpose of the stopping mixture was twofold, to stop the reaction and to protonate the [^{3}H]acetate so that it would extract efficiently into the scintillation mixture. In the original paper on which this assay is based, Potter (4) accomplished these purposes by adding HCl to a final concentration of 0.1 M. We found that this process led to an acid-catalyzed nonenzymatic hydrolysis of acetylcholine at a rate (1.3% per hr) too high for our purposes (see Fig. 1). Consequently we chose instead to add a strong buffer to bring the pH to 2.5, at which point the reaction is stopped, the [^{3}H]acetate is at least 98% protonated, and acid-catalyzed hydrolysis of residual acetylcholine is approximately 100-fold less rapid (see Fig. 1). A convenient buffer for this purpose has been chloroacetic acid, which has an appropriate pK and high solubility.

In order to facilitate extraction of the [^{3}H]acetate, NaCl was also added to increase the polarity of the aqueous phase. That this enhanced

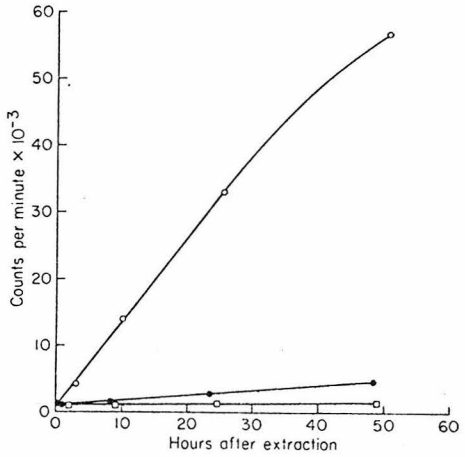

FIG. 1. Effects of various stopping mixtures on the stability of acetylcholine after extraction. One hundred microliters of premixed assay buffer, labeled substrate and H_2O were aliquoted to each of 15 vials; each vial received 10^5 cpm of [^{3}H]acetylcholine and had a final acetylcholine concentration of 10^{-3} M. No enzyme was added. For each of three stopping mixtures, a set of five vials received 0.1 ml of that mixture, plus 4 ml scintillation mixture at time zero, and was counted at various intervals threafter to determine the extent of nonenzymatic hydrolysis of acetylcholine which had occurred. Incubation was at room temperature. The three stopping mixtures were: (–○–) 0.2 M HCl + 2.0 M NaCl; (–●–), 1 M $ClCH_2COOH$ + 2.0 M NaCl; (–□–) 1 M $ClCH_2$ COOH + 0.5 M NaOH + 2.0 M NaCl. The last mixture was chosen for standard use.

TABLE 1
PARTITIONING OF [^{3}H]ACETYLCHOLINE AND [^{3}H]ACETATE BETWEEN AQUEOUS AND TOLUENE PHASES UNDER VARIOUS CONDITIONS[a]

	Radioactively labeled compound	Stopping mixture	Toluene phase volume (ml)	% of initial cpm recovered in		
				Aqueous phase	Toluene phase	Total
1.	[^{3}H]acetate	Standard	4	23.1	71.0	94.1
2.		Standard	4	24.9	72.2	97.1
3.		Standard	10	13.7	80.4	94.1
4.		Standard	10	13.4	84.2	97.6
5.		0.2 M HCl	4	33.0	71.3	104.3
6.		0.2 M HCl, 2 M NaCl	4	22.2	78.5	100.7
7.	[^{3}H]acetylcholine, unhydrolyzed	Standard	4	90.4	0.5	90.9
8.		Standard	4	97.0	0.5	97.5
9.		Standard	4	97.0	0.5	97.5
10.	[^{3}H]-acetylcholine, hydrolyzed	Standard	4	21.5	76.2	97.7
11.		Standard	4	22.6	71.0	93.6
12.		Standard	4	19.6	72.6	92.2
13.		Standard	4	20.6	71.4	92.0
14.		Standard	4	23.1	69.2	92.3
15.		Standard	4	23.6	74.2	97.8
16.		Standard–NaCl	4	30.2	66.8	97.0

[a] [^{3}H]acetylcholine chloride or [^{3}H]sodium acetate (~ 10^5 cpm in either case) was diluted into 0.05 ml of 10^{-3} M unlabeled acetylcholine iodide in a ground glass stoppered 15 ml conical centrifuge tube. 0.05 ml of 0.05 M potassium phosphate buffer, pH 7.0, was added and a 10 min incubation period followed. When acetylcholine was to be hydrolyzed during this period (lines 10–16) the tubes received in addition 0.01 ml of a solution of electric eel cholinesterase (20 units*/ml in 0.01 M Tris buffer, pH 7.4, containing 0.1 mg/ml bovine serum albumin for stability); this was a considerable excess over the enzyme required for complete hydrolysis. After incubation, the tubes received 0.1 ml of the indicated stopping mixture and 4 ml or 10 ml of toluene phase, as indicated; the standard stopping mixture was 1 M $ClCH_2COOH$, 0.5 M NaOH, 2.0 M NaCl, and the toluene phase was 10% isoamyl alcohol in toluene. After thorough shaking and low-speed centrifugation, to separate the phases, each phase was dissolved for counting in 10 ml Bray's dioxane-based scintillation fluid (7) to which had been added complementary unlabeled aliquots of the opposite phase, so that all samples were eventually counted in equivalent solutions.

* 1 unit hydrolyzes 1 μmole of acetylcholine/min at 25°C.

the extraction can be seen from Table 1, lines 10–16. The final stopping mixture, then, contained 1 M chloroacetic acid, 0.5 M NaOH, and 2 M NaCl, and was as effective for acetate extraction as Potter's HCl method (Table 1, lines 1, 2, and 5).

Scintillation Mixture

The scintillation mixture was designed to provide efficient extraction and counting of [^{3}H]acetate without significant counting of [^{3}H]acetylcholine. Following Potter (4) we added isoamyl alcohol in varying

proportions to a standard toluene-based scintillation fluid (0.5% PPO, 0.03% POPOP in toluene), and found that the efficiency of extraction of acetate from an aqueous phase was relatively constant over the range of 10–20% isoamyl alcohol; we chose 10% for reasons of economy. The volume of scintillation mixture to be used was arrived at empirically; as can be seen from Table 1, lines 1–4, increasing the volume from the standard 4 ml up to 10 ml provided a slightly higher efficiency of extraction for [^{3}H]acetate, but for normal use this was judged to be an insufficient increase to justify the added expense. With the standard 4 ml of scintillation mixture and the standard stopping mixture, as shown in Table 1, lines 7–15, only 0.5% of the unhydrolyzed [^{3}H]acetylcholine is counted, whereas 70–75% of of the [^{3}H]acetate released by hydrolysis is counted.

RESULTS

When acetylcholinesterase from a variety of sources was measured with the Standard Assay Procedure described above, the activity measurements were reproducible, linearly dependent on time and on enzyme concentration, and comparable with the values obtained by a commonly used colorimetric assay. These points are demonstrated by the assays of electric eel (*Electrophorus electricus*) acetylcholinesterase described below.

Reproducibility. Twenty duplicate 15-min measurements were made on a given sample of electric eel acetylcholinesterase at a substrate concentration of 2×10^{-3} M. The mean value and standard deviation for these measurements was $15{,}813 \pm 213$ cpm (representing 15.8% hydrolysis of the substrate). Since these samples were counted for 5 min each (av total counts 79,065), the expected standard deviation from statistical counting error alone is ± 58 cpm. The additional error is comparable to the nominal error of the automatic pipettors used ($\pm 1\%$).

Linearity with time and enzyme concentration. Figure 2a and 2b show the results of assays of two different amounts of electric eel acetylcholinesterase over the time ranges 0–30 min and 0–3 hr. In each instance, the amount of hydrolysis increased linearly with time. In other experiments this linearity was found to hold for even longer times, provided that hydrolysis did not exceed 30% (30,000 cpm). The nonenzymatic contribution to this hydrolysis was extremely slight (0.23%/hr), as shown in Fig. 2b, and the constancy of the enzymatic hydrolysis rate over long times shows that electric eel acetylcholinesterase was stable under assay conditions. Figure 2c shows that the enzyme activity measured by these experiments was, as expected, directly proportional to the amount of enzyme added.

Comparison with a colorimetric assay. The commonly used colorimetric assay of Ellman *et al.* (8) was used as a standard against which to

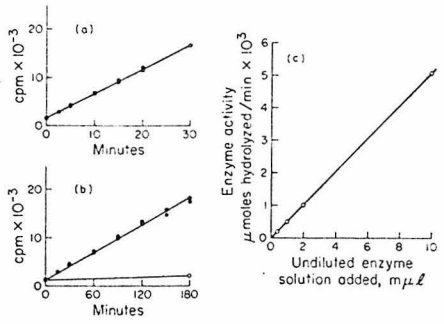

FIG. 2a and 2b. Time dependence of the assay. A solution of electric eel eel acetylcholinesterase nominally 200 units*/ml in 0.01 M Tris buffer, pH 7.4, containing 0.1 mg/ml bovine serum albumin (BSA) for stability, was diluted as indicated into 0.01 M Tris buffer, pH 7.4, 0.1 mg/ml BSA. Dilution for (a) was 5000-fold, for (b) 25,000-fold. Ten microliters of the diluted enzyme were incubated at room temperature for the indicated times with 0.05 ml 0.005 M potassium phosphate buffer, pH 7.0, 0.02 ml 10^{-2} M ^{3}H-labeled acetylcholine substrate, and 0.02 ml H_2O. Reactions were stopped with 0.1 ml of standard stopping mixture and 4 ml scintillation mixture and counted in a Beckman LS 200-B scintillation counter. (–○–) Blank, without enzyme; (–●–) reactions, with enzyme. All assays were performed in duplicate, and both values are plotted; overlap sometimes obscures the second point. 2c—Concentration dependence of the assay. From the experiments of Figs. 2a and 2b and other experiments of similar design, measured enzyme activities were calculated from the slopes of the kinetic curves, according to the following formula:

$$\text{Acetylcholinesterase activity} = \mu\text{moles acetylcholine hydrolyzed/min}$$

$$= \text{slope (in cpm/min)} \times \frac{\text{acetylcholine conc. (in } \mu\text{moles/ml)} \times 0.1 \text{ ml}}{\text{cpm after total hydrolysis}}$$

The value of a no-enzyme blank was subtracted from all cpm values, and total hydrolysis was accomplished by adding (to a separate vial) 10 μl of an electric eel acetylcholinesterase solution, 100 units*/ml. The calculated activities are plotted here against the amount of added enzyme.

* 1 unit hydrolyzes 1 μmole of acetylcholine/min at 25°C.

compare the radiometric assay. A sample of electric eel acetylcholinesterase was assayed several times with both assays, as shown in Table 2. The comparison of assay values is complicated because: (1) the colorimetric assay uses acetylthiocholine instead of acetylcholine as substrate, and (2) the colorimetric assay is usually performed at the pH optimum of 8.0. Nonetheless the values obtained when both assays are performed at pH 7.0 are very close, substantiating the validity of the radiometric assay. (The radiometric assay, like the colorimetric one, can be made about 50% more sensitive by raising the pH to 8.0, but this sensitivity gain is accompanied in both cases by a much more extensive rise in the background due to nonenzymatic hydrolysis. In practice, this background rise constitutes a serious limitation when using the assay to detect low activity levels, and assays at pH 7.0 are preferable.)

TABLE 2
COMPARISON OF RADIOMETRIC AND COLORIMETRIC ASSAYS[a]

Assay	pH	Activity ($\times 10^2$ μmole/min/ml)
Colorimetric	8.0	4.32 ± 0.18 (6)
Colorimetric	7.0	2.84 ± 0.08 (7)
Radiometric	7.0	3.17 ± 0.17 (5)
Radiometric	7.0	3.25 ± 0.14 (9)

[a] Electric eel acetylcholinesterase (P-L Biochemicals lot no. 461-2) was dissolved at 400 units*/ml in 0.01 M sodium phosphate buffer, pH 7.0, 0.1 M NaCl, and diluted 1000-fold in the same solution + 1 mg/ml bovine serum albumin (BSA for stability). The colorimetric assay at pH 8.0 was performed by mixing 2.80 ml of 0.1 M sodium phosphate buffer pH 8.0, 0.1 ml of 0.06 M acetylthiocholine, 0.10 ml of 0.01 M 3,3′ dithiobis[6-nitrobenzoic acid] (DTNB; dissolved in 0.1 M sodium phosphate buffer as described by Ellman [8]), and 0.15 ml of the enzyme dilution. The optical density change at 412 nm was followed in a Beckman Acta III recording spectrophotometer. The colorimetric assay at pH 7.0 was identical except that the buffer was 2.80 ml of 0.0232 M sodium phosphate buffer, pH 7.0. Measured rates of optical density change were converted to activities using the conversion factors of Ellman *et al.* (8). The radiometric assays were performed as described in the text, using 2×10^{-3} M acetylcholine substrate, 15 min incubations, and 0.005 ml of the enzyme dilution. These conditions led to 10–11% hydrolysis of the substrate. To avoid any possible complications from loss of enzyme activity, one group of radiometric assays (line 3) was performed before the colorimetric assays, and a second group (line 4) immediately thereafter; the two groups do not differ significantly. Enzyme activities were calculated for the radiometric assays using the formula of Fig. 2c. All activity values are given as the mean ± standard deviation, and the number of replicate assays is included in parentheses.

* 1 unit hydrolyzes 1 μmole of acetylcholine in 1 min at 25°C.

DISCUSSION

In the radiometric cholinesterase assay described above, [^{3}H]acetylcholine is hydrolyzed in a scintillation vial containing a small reaction volume, from which the [^{3}H]acetate produced by hydrolysis is extracted into a toluene-based scintillation fluid added directly to the vial. The [^{3}H]acetate is then measured simply by counting the whole vial without further manipulations. There is no need to remove the unhydrolyzed [^{3}H]acetylcholine; it remains uncounted in the small aqueous reaction volume, from which the weak β-particles of its ^{3}H-decay do not escape to reach the scintillator. (The same principle, viz. selective counting of a ^{3}H-labeled product by extraction into an organic scintillation fluid, has been used by Roffman, Sanocka, and Troll (9) to assay proteolytic enzymes such as trypsin in a sensitive and convenient fashion.) The assay differs from that of Potter (4) in relatively minor ways. First, it uses ^{3}H as opposed to ^{14}C substrate, which allows it to circumvent Potter's final postassay extraction step and to use the scintillation vial as reaction

vessel. And second, it uses a buffered stopping solution, which prevents postassay acid-catalyzed hydrolysis and allows stopped samples to be stored for relatively long times before counting. Although these modifications are relatively minor, we have found in practice that the convenience they afford has made a considerable difference in the ease and willingness with which the assay of large numbers of replicate samples is undertaken.

The assay depends for its convenience on the availability of (^{3}H-acetyl)-choline chloride; (^{14}C-acetyl)-choline chloride will not work because the ^{14}C-decay produces a more energetic β-particle which too frequently escapes from the aqueous phase and excites the scintillator. In principle the assay should be easily adaptable to the use of other (acyl-^{3}H)-choline substrates, but since none of these is currently available commercially, the assay is not currently well suited for studies of substrate specificity. However, it is well suited for inhibitor studies without further modification.

Because of its radiometric nature and the relatively high specific radioactivity of available (^{3}H-acetyl)-choline chloride, the assay [like the assay of Potter (4)] can be made extremely sensitive; we have used it, for example, to measure the acetylcholinesterase activities of individual nematodes of the species *Caenorhabditis elegans* (total wet wt ~ 1 μg). Because it is by nature an endpoint measurement, it is not as well suited as some colorimetric methods for kinetic studies on relatively large amounts of enzyme; however, its sensitivity and convenience make it useful for multiple-time-point kinetic studies on smaller amounts of enzymes not assayable by the less sensitive colorimetric methods.

The assay has been in routine use in our laboratory and in some others for several years and has proved accurate and reliable for the measurement of cholinesterase activities from a variety of sources, including rat diaphragm (10), *Torpedo californica* electric organ (11), and, in our own hands, *Electrophorus electricus* electric organ, *Bungarus multicinctus* venom, *Drosophila melanogaster* heads, horse serum, bovine erythrocytes, and extracts of the small soil nematode *Caenorhabditis elegans*.

ACKNOWLEDGMENT

We thank Dr. Sydney Brenner for suggesting some of the ideas developed in this assay.

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II Separation and Selective Modification of *Electrophorus electricus* Acetylcholinesterases by Collagenase

CARL D. JOHNSON, SUSAN P. SMITH and
RICHARD L. RUSSELL

Abstract

The multiple forms of acetylcholinesterase found in the electric organ of the eel *Electrophorus electricus* have been fractionated by differential solubilization from an ammonium sulfate precipitate, using a column elution procedure (King, 1972). This procedure cleanly separates "native" forms from "degraded" forms, and subsequent sedimentation reveals three native and two degraded forms. All three native forms, in distinction to the degraded ones, are insoluble at low ionic strength and are shifted to higher sedimentation constants by limited collagenase treatment. These results suggest that the long (500 Å) tail seen previously on the native forms of this enzyme (Dudai, Herzberg & Silman, 1973; Rieger *et al.*, 1973) may contain collagen.

Introduction

Multiple molecular forms of the enzyme acetylcholinesterase (EC 3.1.1.7) have been observed in extracts from various sources, including the enzyme-rich electric organs of *Electrophorus electricus* and *Torpedo marmorata* (Massoulie & Rieger, 1969). In *Electrophorus electricus*, various conditions for extraction of these forms have been tested, revealing that most of the native activity requires high ionic strength for its extraction (Silman & Karlin, 1967; Massoulie & Rieger, 1969; Dudai & Silman, 1974[a]), and that the extracted activity is primarily in three "native" forms with sedimentation constants of 9 S, 14 S, and 18 S (Massoulie & Rieger, 1969). Preparations of all three native forms show a common electron microscopic structure, consisting of a globular portion attached to an elongated "tail" (Dudai, Herzberg & Silman, 1973; Rieger *et al.*, 1973), and it has been suggested that the tail might serve to attach the enzyme to the synaptic area. The three forms differ in the size of the globular portion and apparently contain one, two, and three copies, respectively, of a globular unit, which itself appears tetrameric by electron microscopy (Rieger *et al.*, 1973).

Two additional forms of acetylcholinesterase are also found in *Electrophorus electricus* extracts; in fresh extracts these represent a minority of the total activity, but they become quantitatively more important in aged

extracts (where autolysis has had a chance to occur) or in extracts deliberately sonicated or treated with non-specific proteases (Massoulie, Rieger & Tsuji, 1970; Massoulie, Rieger & Bon, 1971). One of these forms, with a sedimentation constant of about 12 S, is the predominant form in commercially available Electrophorus electricus enzyme (prepared autolytically, Leuzinger et al., 1968). It lacks the characteristic tail of the native forms, and apparently consists exclusively of the globular tetrameric unit (Rieger et al., 1973). The second form also lacks the tail and apparently consists of a globular dimer (Rieger et al., 1973).

In this paper we describe a solubility-based fractionation procedure which cleanly separates the "native" tailed forms of Electrophorus electricus acetylcholinesterase, as a group, from the "degraded" globular forms. This procedure eases the previously difficult task of separating the smallest tailed form (9 S) from the dimeric globular form (7.5 S). The clean separations achieved permit the forms to be separately characterized. The tailed and globular forms show the solubility differences expected from previous work, and in addition collagenase selectively affects the tailed forms, suggesting a collagen-like component in the tail.

Materials

The detergents Triton X-100 and Tween 80, the proteins bovine serum albumin (BSA, Fraction V), yeast alcohol dehydrogenase (ADH), E. coli β-galactosidase (gal), bovine catalase (cat), electric eel acetylcholinesterase (AChE), Clostridium welchii phospholipase C, and the chemicals acetylcholine (ACh) iodide, PPO and POPOP were purchased from Sigma Chemical Co. Trypsin (TRLOGC) was from Worthington, Celite 545 was from Fisher Chemical Co. Ammonium sulfate (enzyme grade) was from Schwarz-Mann. Chemicals for polyacrylamide gel electrophoresis were obtained from BioRad Laboratories. Renografin (Squibb) was obtained from a local apothecary. [^{3}H]-acetylcholine was TRA277 from Amersham-Searle, or NET-113 from New England Nuclear. Diisopropyl-1-[^{3}H]-fluorophosphate ([^{3}H]-DFP) was NET-065 from New England Nuclear. Clostridium histolyticum collagenase was obtained from two sources, Worthington Biochemical Corp. (CLSPA) and Advanced Biofactures Corp. (Form III).

Buffers were dilutions of phosphate, pH 7.0 (0.05 M KH_2PO_4-K_2HPO_4); borate, pH 8.8 (0.1 M $Na_2B_4O_7$, adjusted with HCl); and Tris, pH 7.5 (0.1 M tris (hydroxymethyl) amino methane, adjusted with HCl).

Methods

Cholinesterase assays were performed by a radiometric liquid extraction assay (Johnson & Russell, 1975). Assays were buffered at pH 7.0 with 0.025 M phosphate and included 5×10^{-5} M or 2×10^{-3} M acetylcholine and 0.1 µC or less [^{3}H]-ACh in 100 µl. Assays were started by the addition of enzyme and stopped by the addition of 100 µl of 1 M $ClCH_2COOH$, 0.5 M NaOH, 2 M NaCl; then 4.0 ml of a standard toluene scintillation fluid (0.5% PPO, 0.03% POPOP) with 10% isoamyl alcohol or butyl alcohol was added, and the vials were capped, shaken vigorously and counted (the released [^{3}H]-acetate extracts into the toluene phase and counts selectively; see Johnson & Russell, 1975). Most sets of assays included several blanks (without enzyme) and several "complete conversions" (including 10 µl of 100 u/ml commercial AChE preparation to effect complete hydrolysis of the [^{3}H]-ACh and thereby determine the maximum number of cpm to be expected). With occasional unimportant exceptions, all determinations were within the linear range of the assay ($\geq$ 35% conversion), and are expressed as cpm of extracted [^{3}H]-acetate, with blank subtracted.

Electric eels were obtained from local suppliers. Usually the animals were killed by cooling to 0 C° for 30 min, and the electric organs were then removed, flash-frozen in liquid nitrogen and stored at -20 C° until use.

Aliquots of electric organ were homogenized at 0°C in 5-10 volumes of 10% sucrose, 0.005 M $MgCl_2$ (H.K. Mitchell, personal communication) for 2 times 1 minute using a Sorvall Omni-Mixer. The homogenate was spun at 20,000 rpm for 30 min in a Sorvall centrifuge. The supernatant contains only 5-10% of the activity. The pellet was extracted by rehomogenizing in 2-5 volumes of 0.02 M borate, pH 8.8; 1 M NaCl. The 20,000 rpm, 30 min supernatant of this extract contains greater than 80% of the activity and is the source of enzyme for subsequent separation of multiple molecular forms. Earlier experiments at pH 7.0 yielded comparable forms of AChE activity, but since higher pH preparations appear to be more stable, most of the experiments reported are at pH 8.8.

Acetylcholinesterase activity in the supernatant of the 1 M NaCl extract was fractionated by a procedure which depends on differential solubility in concentrated ammonium sulfate solutions (King, 1972). Saturated ammonium sulfate was added to 20% saturation, and a 20,000 rpm, 30 min supernatant was prepared; 4 ml of washed, de-fined and settled Celite suspension was added per 100 ml volume of supernatant and then saturated ammonium sulfate was added to 50% saturation. After gentle stirring for several hours, the Celite was collected by settling, poured into a column, and eluted with a gradient from 50% to 0% saturated ammonium sulfate in 0.02 M borate, pH 8.8; 1 M NaCl.

The multiple molecular forms were separated by velocity sedimentation in 5-20% sucrose density gradients (Martin & Ames, 1961). A non-ionic detergent and additional protein were added to some gradients to prevent the large losses of activity seen with dilute enzyme; they do not affect sedimentation velocities.

When gradients having different numbers of fractions were to be compared, activity was plotted against "fractional position" in the gradient, calculated with the formula: fractional position = 1 -

$$\frac{\text{fraction number} - 0.5}{\text{Total fractions}} \quad 1 - \frac{0.5 \times \text{sample volume}}{\text{gradient volume} + \text{sample volume}}$$

For SDS-polyacrylamide gel electrophoresis, samples were diluted with one volume of 0.02 M (Na) phosphate, pH 7.0, 10% sucrose, 2% SDS, 0.005 M dithiothreitol, 0.01% bromophenol blue and heated for 5 min in boiling water. A linear gradient slab gel (15 cm x 11 cm x 0.1 cm thick) from 18.5 to 7.5% polyacrylamide was used for separation. A 3% polyacrylamide stacking gel was layered on top of the separation gel and a removable comb was used to form sample wells. The buffers for separation and stacking gels and the running buffer were those of Laemmli (Laemmli, 1970). Electrophoresis was carried out with a current of 20 mA until 30 min after the bromophenol blue marker reached the bottom of the gel. Proteins were fixed

and stained in 10% acetic acid, 10% methanol, 0.1 % Coomassie brilliant blue, then diffusion destained in the same solvent. When several samples were to be compared, they were run in adjacent wells of the same gel.

Collagenase digestions were performed at room temperature in Tris, pH 7.5. Two commercial collagenase preparations were used. Worthington collagenase (CLSPA, 400 u/mg) was usually used at 10 µg/ml. A several hundred-fold excess of BSA was added to inhibit nonspecific proteolytic effects. The Advanced Biofactures collagenase (Form III nominally 2500 u/ml) was usually used at a 100-fold dilution. This preparation is ostensibly uncontaminated with nonspecific proteases (Miller & Udenfriend, 1970).

Results

Homogenates of Electrophorus electricus electric organ, when prepared by the high pH, high ionic strength extraction method described in Materials & Methods, contain at least three major forms of soluble acetylcholinesterase which can be separated by velocity sedimentation, as previously described (Massoulie & Rieger, 1969) and as shown in Figure 1. Further resolution of these forms can be accomplished by differential ammonium sulfate solubility; the activity of an extract is precipitated onto a solid Celite support at high

Fig. 1. Sedimentation of homogenate. An aliquot of electric organ homogenate was diluted into 20 volumes of 0.02 M borate, pH 8.8; 1 M NaCl; 1% Triton X-100. Fifty µl was loaded onto 4.5 ml 5-20% sucrose gradients in 0.02 M borate; 1 M NaCl; 0.1% Tween 80; 1 mg/ml BSA with 0.5 ml 100% Renografin cushions (shaded area). Gradients were centrifuged for 3.5 hr at 65,000 rpm. 5-drop fractions were collected. Ten µl of each fraction was assayed for 5 min with 5×10^{-5} M ACh. Complete conversion yielded 19.9×10^{3} cpm, the blanks gave 1.1×10^{3} cpm. Enzymatic activity is plotted versus fraction number. Sedimentation is from right to left.

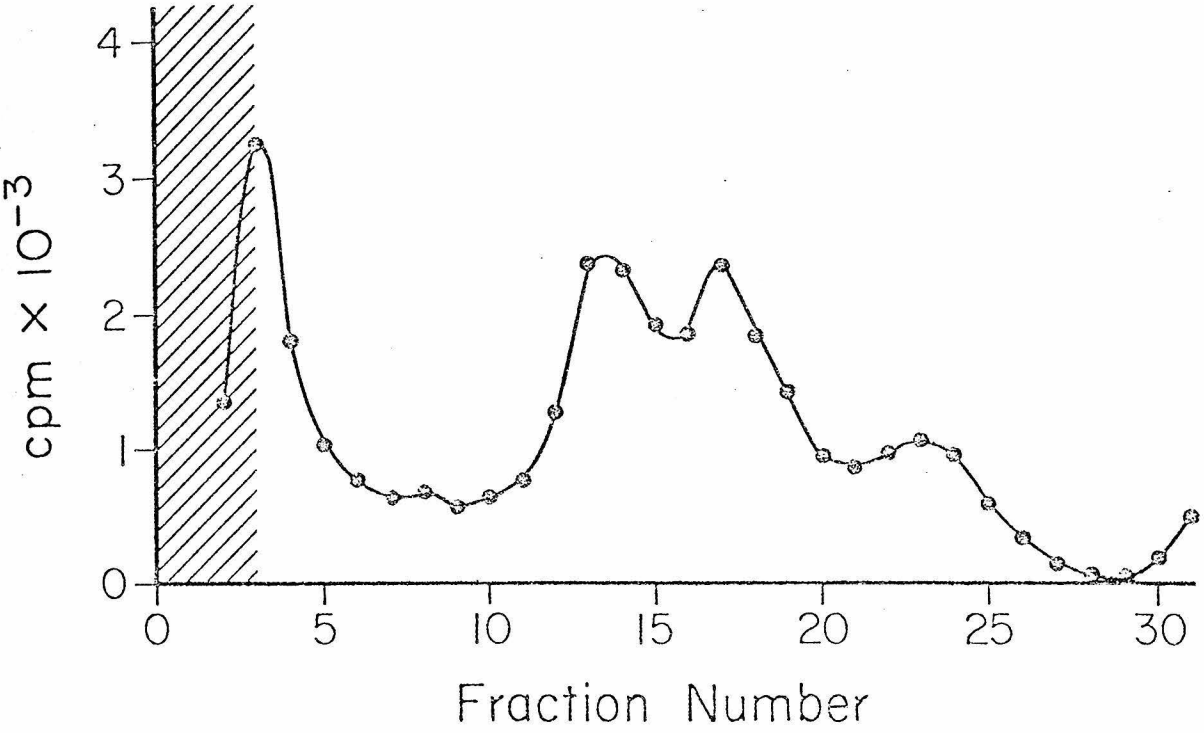

Figure 1

ammonium sulfate concentration and then eluted with a gradient of decreasing ammonium sulfate concentration, as described in Materials & Methods. As shown in Figure 2A, the activity elutes as three distinct peaks. Upon subsequent fractionation by velocity sedimentation, these peaks prove to have different compositions, as shown in Figure 2B. The small initial peak (peak 1) contains the same forms, in essentially the same proportions, as the original extract. Its origin is unclear, and it was not seen in all runs. The intermediate peak (peak 2) contains two forms, whose properties show them to be the globular tetramer and dimer previously identified (see below). The third, and by far the most prominent peak of the ammonium sulfate elution pattern (peak 3) contains three different forms whose properties show them to be the "native" tailed forms previously identified (see below).

Each of the five forms appearing in peaks 2 and 3 of the ammonium sulfate elution pattern can be isolated and resedimented as a single discrete peak, and the results described below show that each is a distinct entity. Accordingly, and to avoid confusion with previous nomenclature, these forms are subsequently referred to by the Roman numeral designations I-V, as indicated in Figure 2.

When the separated forms I-V are isolated and

Fig. 2. Fractionation of AChE on Celite-ammonium sulfate columns. From 11.5 g of electric organ tissue a 1 M NaCl extract was prepared and fractionated on a Celite-ammonium sulfate column, as described in Materials and Methods. A 50 ml volume of extract was precipitated onto 2 ml of settled Celite, poured into a 1 cm diameter column and eluted with 100 ml of a 50%-0% ammonium sulfate gradient. A. Five µl of each fraction was assayed for 30 sec with 2×10^{-3} M ACh. Complete conversion gave 110.6×10^{3} cpm, and blanks gave 3.3×10^{3} cpm. Three sets of fractions, representing the 1st (fractions 19-21), 2nd (fractions 24-27) and 3rd (fractions 31-45) peaks were separately pooled and reprecipitated by addition of saturated ammonium sulfate to 50% saturation. The resulting pellets were redissolved in 0.02 M borate, pH 8.8; 1 M NaCl. B. Aliquots of peaks 1, 2, and 3 were loaded onto 5 ml 5-20% sucrose gradients in 0.02 M borate, pH 8.8; 1 M NaCl; 0.1% Tween 80. Gradients were centrifuged at 65,000 rpm for 4.5 hr. Five-drop fractions were collected and 10 µl of each fraction was assayed for 30 sec with 2×10^{-3} M ACh. Complete conversion yielded 112.3×10^{3} cpm; the blank gave 3.4×10^{3} cpm. Enzymatic activity is plotted against fractional position.

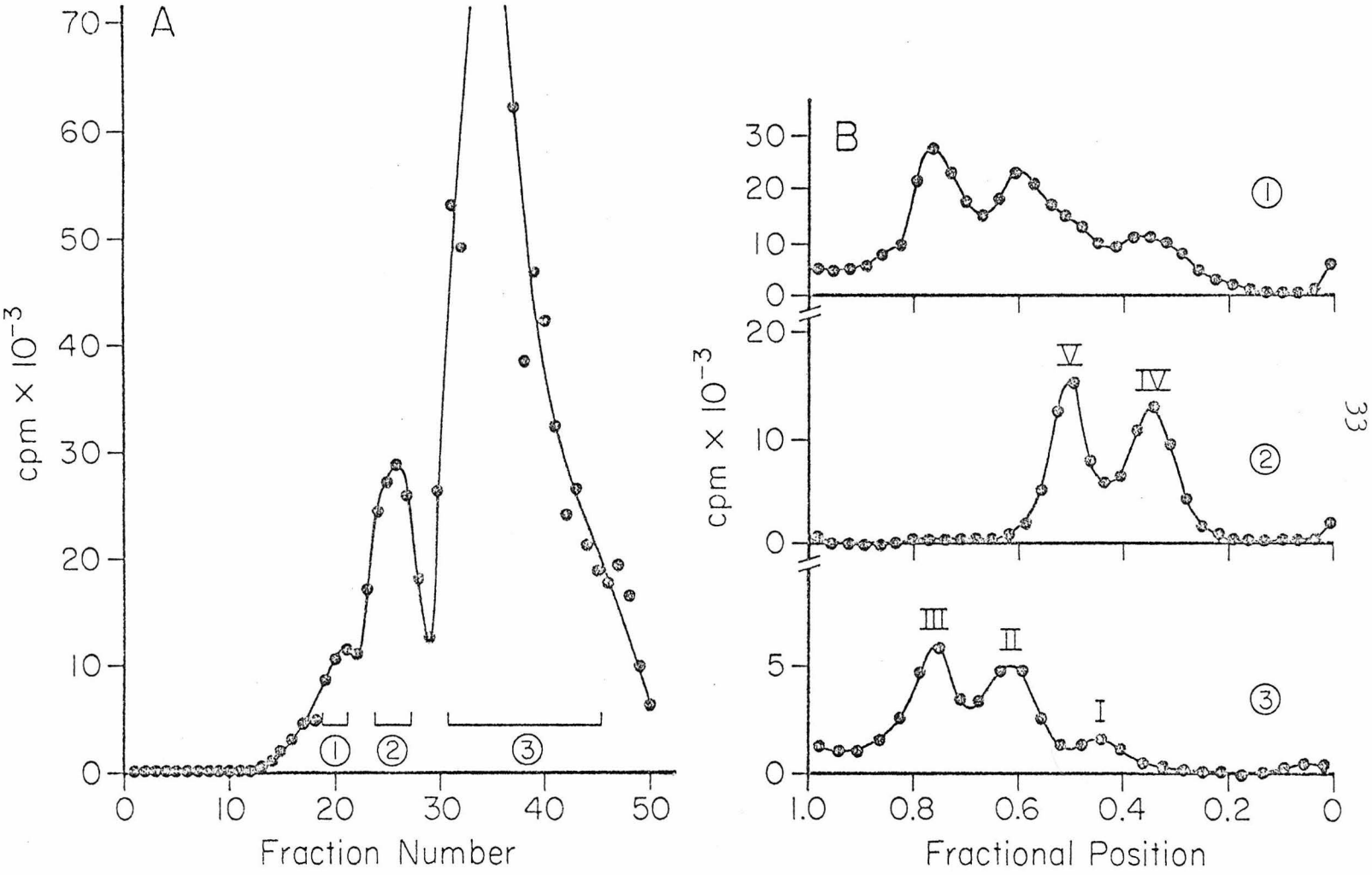
A
B
cpm × 10⁻³
Fraction Number
Fractional Position
0
10
20
30
40
50
60
70
5
1.0
0.8
0.6
0.4
0.2
①
②
③
I
II
III
IV
V

Figure 2

resedimented without the 1 M NaCl used for their extraction, the globular forms IV and V remain soluble at low ionic strength and again resediment as discrete uniform peaks, whereas the tailed forms I, II, and III precipitate and therefore disappear from the gradient (Figure 3). Similar results have been reported previously for forms II, III and V; however, the situation for forms I and IV has been more confusing, due to the fact that they have not been reliably resolved. In particular, the presence of significant amounts of form IV in preparations presumed to consist mostly of the native form I has led to the erroneous conclusion that form I is soluble at low ionic strength (Massoulie & Rieger, 1969; Dudai & Silman, 1974a). In view of this precedent, a careful solubility study of form I was undertaken, as shown in Figure 4; form I clearly shows the low-ionic-strength insolubility characteristic of forms II and III.

Previous electron microscopic studies of forms I, II and III have shown the presence of a $\sim$500 Å tail in addition to globular subunits (Dudai et al., 1973; Rieger et al., 1973). The possibility that these tails contain collagen is suggested by the observation that a large form of acetylcholinesterase (perhaps homologous with form II or III) can be selectively removed from rat diaphragm endplates with collagenase (Hall, 1973). Accordingly the effects of collagenase on forms I-V

Fig. 3. Sedimentation of separated forms I, II, III in 1 M NaCl and in no NaCl. Separated forms I, II, III were diluted into 0.05 M phosphate, pH 7.0 with (A) or without (B) 1 M NaCl. One hundred-µl aliquots were loaded onto 5.2 ml 5-20% sucrose gradients in the same buffer. Gradients were centrifuged at 65,000 rpm for either 4.5 hr (A) or 4.0 hr (B). Fractions were collected and 10 µl (A) or 20 µl (B) of each was assayed for 15 min with 2×10^{-3} M ACh. Complete conversion yielded $\sim 100 \times 10^3$ cpm. Blank was 1.6×10^3 cpm (A), or 1.3×10^3 cpm (B). Activity is plotted against fractional position.

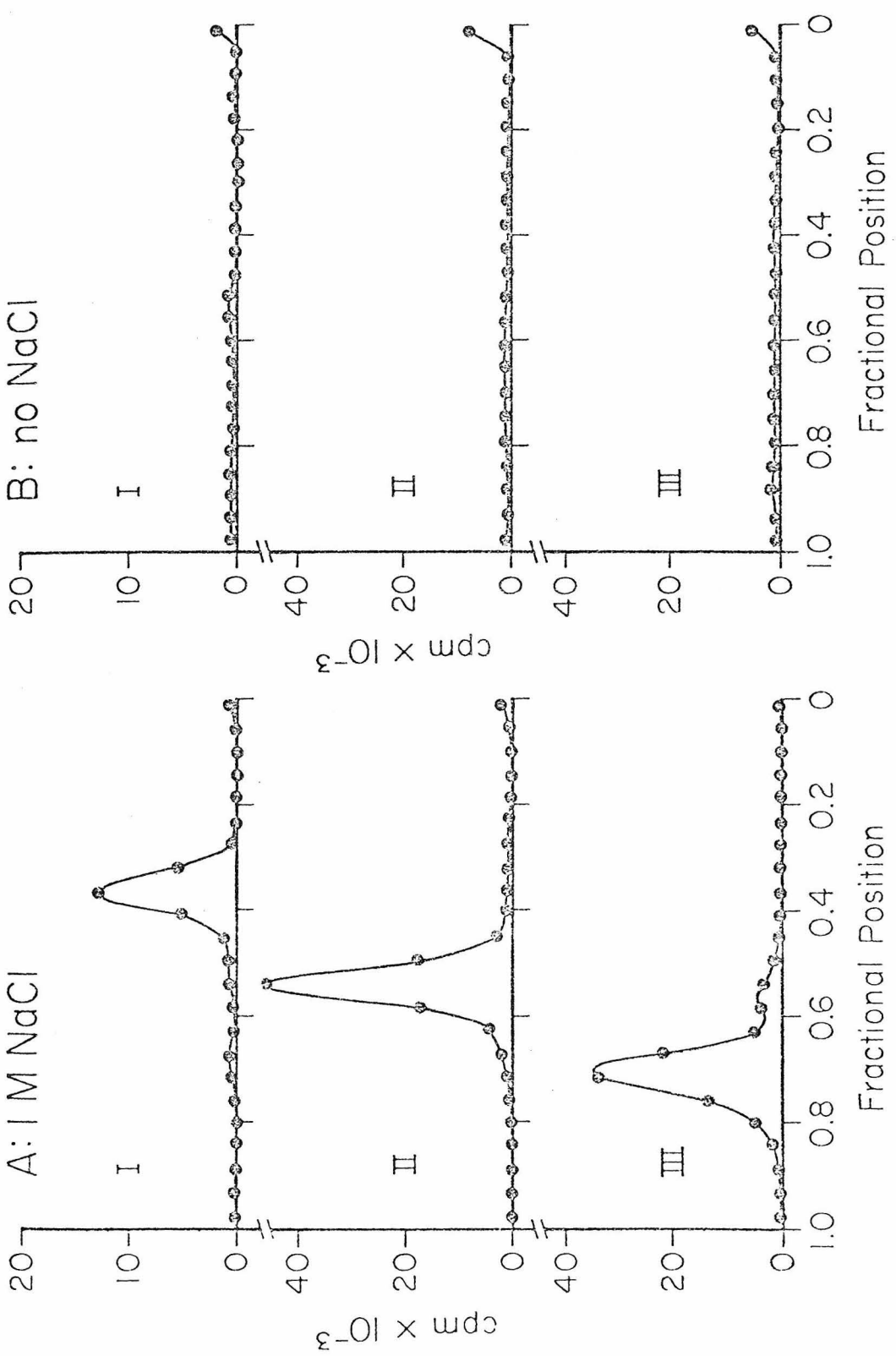
A: 1 M NaCl
B: no NaCl
I
II
III
cpm x 10⁻³
Fractional Position
20
10
0
40
20
0
1.0
0.8
0.6
0.4
0.2
0

Figure 3

Fig. 4. Sedimentation of form I as a function of NaCl concentration. AChE form I, prepared in 0.05 M phosphate, pH 7.0; 1 M NaCl, was diluted with 1 volume of 0.05 M phosphate; 0.01% Triton X-100. One or two hundred-µl aliquots were loaded onto nine different 5-20% sucrose gradients containing NaCl concentrations from 0.1 M to 1 M. After centrifugation at 65,000 rpm for 4.5 hr, fractions were collected. Twenty µl of each fraction was assayed for 20 min with 2×10^{-3} M ACh. In the main figure the percent of activity recovered in the peak (⊛——⊛) and that recovered in the entire gradient (x——x) is plotted as a function of NaCl concentration in the gradient. In the insert, the fractional position of the activity peak is plotted as a function of NaCl concentration; no peak occurs below 0.2 M NaCl.

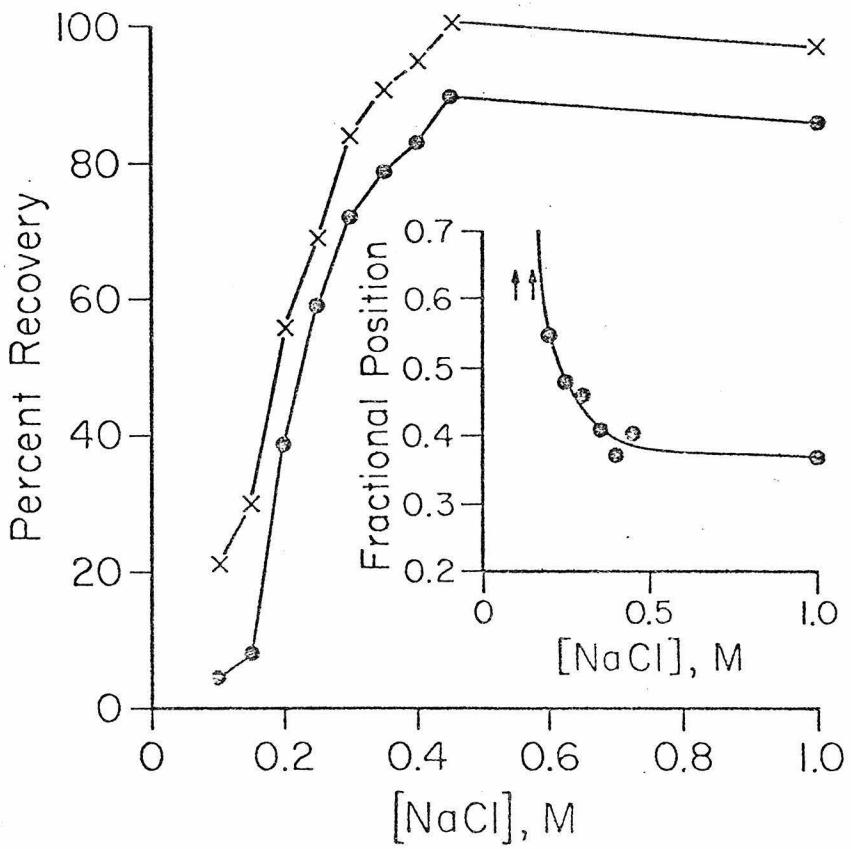
Percent Recovery
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
[NaCl], M
Fractional Position
0.7
0.6
0.5
0.4
0.3
0.2
0
0.5
1.0
[NaCl], M

Figure 4

were investigated. Two different collagenase preparations were used at low concentration, and high levels of BSA were added to try to circumvent the effects of any contaminating nonspecific proteases. When careful sedimentation analysis was carried out, using gradients with sedimentation markers, small but reproducible effects of collagenase treatment were seen, as shown in Figure 5. Forms I, II, and III were all shifted to slightly higher sedimentation constants, while forms IV and V were unaffected.

To control for the possibility that differential collagenase effects on the forms might result from unintended variations in the separate digestions used, a mixture of forms II, III and V was treated with collagenase; as shown in Figure 6, forms II and III were shifted in the same digestion which left form V unchanged. A statistical analysis of a larger series of similar experiments, presented in Table 1, serves to verify the differential nature of the collagenase effect.

Although collagenase selectively affected the (tailed) forms I, II, and III, it did not alter their characteristic low-ionic-strength insolubility (at least for most of the affected molecules) as shown in Figure 7. A small fraction of collagenase-treated forms II-III molecules were rendered soluble in low ionic strength (Figure 7); these molecules sediment with even greater

Fig. 5. Effect of collagenase on sedimentation of separated forms of AChE. Separated AChE forms I, II, III, IV, and V were diluted 10-20X into 100 µl of 0.1 M Tris, pH 7.5. Ten µl of 25 mg/ml BSA was added to each incubation. Control incubations (—●—) received an additional 10 µl of 0.1 M Tris, pH 7.5. Collagenase incubations (—o—) received 10 µl of 100 µg/ml collagenase (Worthington CLSPA). After 1 hr incubation at room temperature, 100-µl aliquots were loaded onto 5.0 ml 5-20% sucrose gradients in 0.02 M borate, pH 8.8; 1 M NaCl; 0.1% Tween 80; 1 mg/ml BSA. β-Galactosidase and ADH were added as markers. Gradients were centrifuged for 5 hr (A) or for 7 hr (B) at 65,000 rpm. Five-drop fractions were collected and 10 µl of each was assayed with 2×10^{-3} M ACh. Complete conversion yielded 100×10^3 (form I) or $\sim 20 \times 10^3$ cpm (forms II-V). Blanks were 5.5×10^3 cpm (form I) or 1.1×10^3 cpm (forms II-V).

Panel A shows the results for forms II, III, and V; panel B shows the results for forms I and IV, including a small amount of contaminating form II. For clarity, only the regions of the activity peaks are plotted. Slight variations in the positions of the markers have been compensated by shifting the fractional position scales

(by 0.025 or less) so that the most relevant marker (gal for A, ADH for B) has the same fractional position in all gradients. Marker positions are indicated by arrows, ↑ for control gradients, ↓ for collagenase gradients.

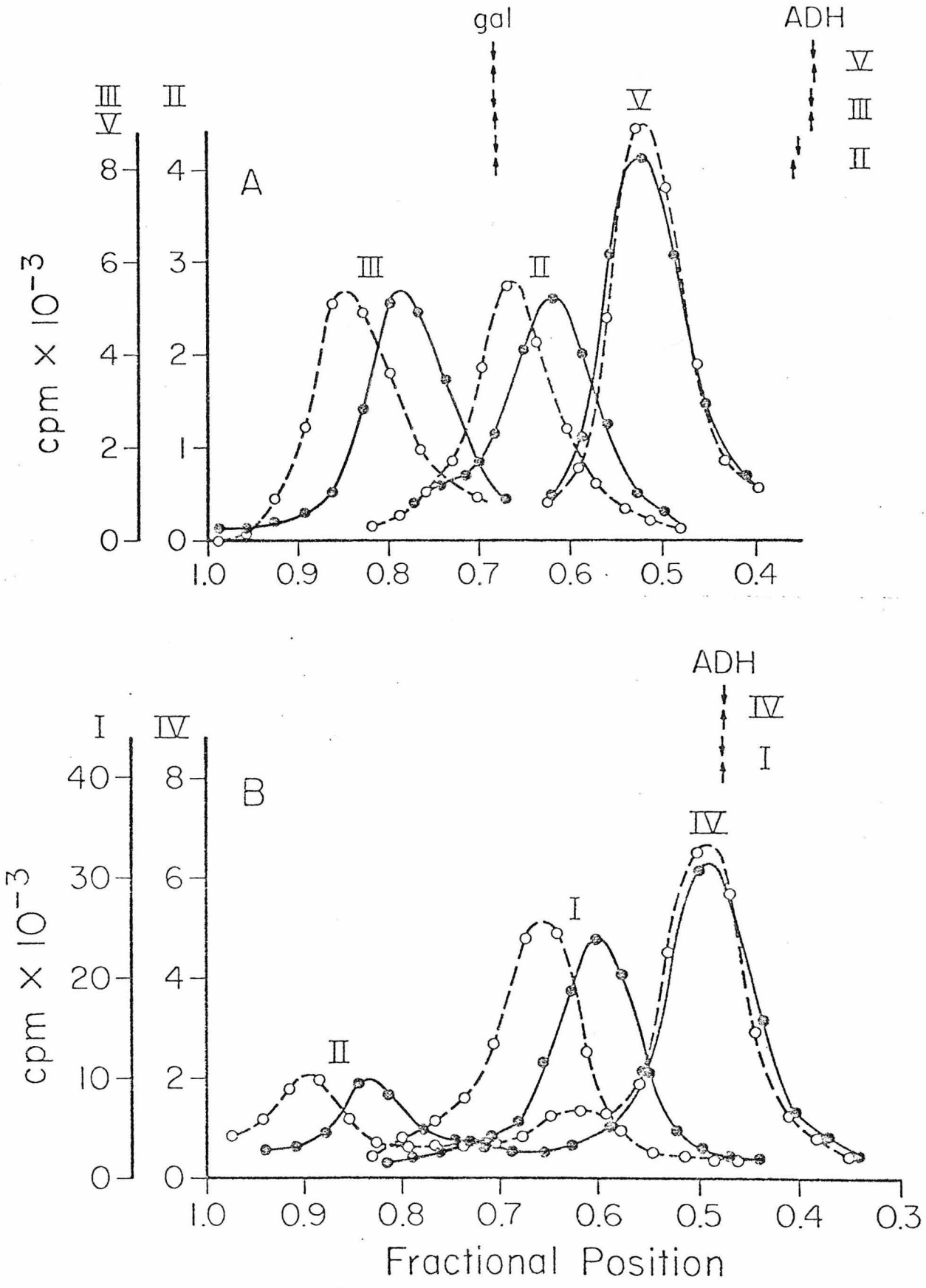

Figure 5

Fig. 6. Effect of collagenase on sedimentation of mixed forms II, III and V. Ten µl of an AChE preparation containing significant amounts of forms II, III, and V was diluted with 10 µl of 0.1 M Tris, pH 7.5; 10 mg/ml BSA and 10 µl of purified collagenase solution (Advance Biofactures Corp.; collagenase form III diluted 1:100 in 0.1 M Tris, pH 7.5) (—●—) or 10 µl of additional Tris buffer (---o---). After 14.5 hr incubation at room temperature, 5-µl aliquots were loaded onto 5.0 ml, 5-20% sucrose gradients in 0.02 M borate, pH 8.8; 1 M NaCl; 0.1% Tween 80; 1 mg/ml BSA. Gradients were centrifuged for 4.5 hr at 65,000 rpm. Five-drop fractions were collected and 2 µl of each fraction was assayed for 3 min with 2×10^{-3} M ACh. Complete conversion yielded 22.2×10^{3} cpm. The blank was 0.5×10^{3} cpm. The marker is indicated as in Fig. 5.

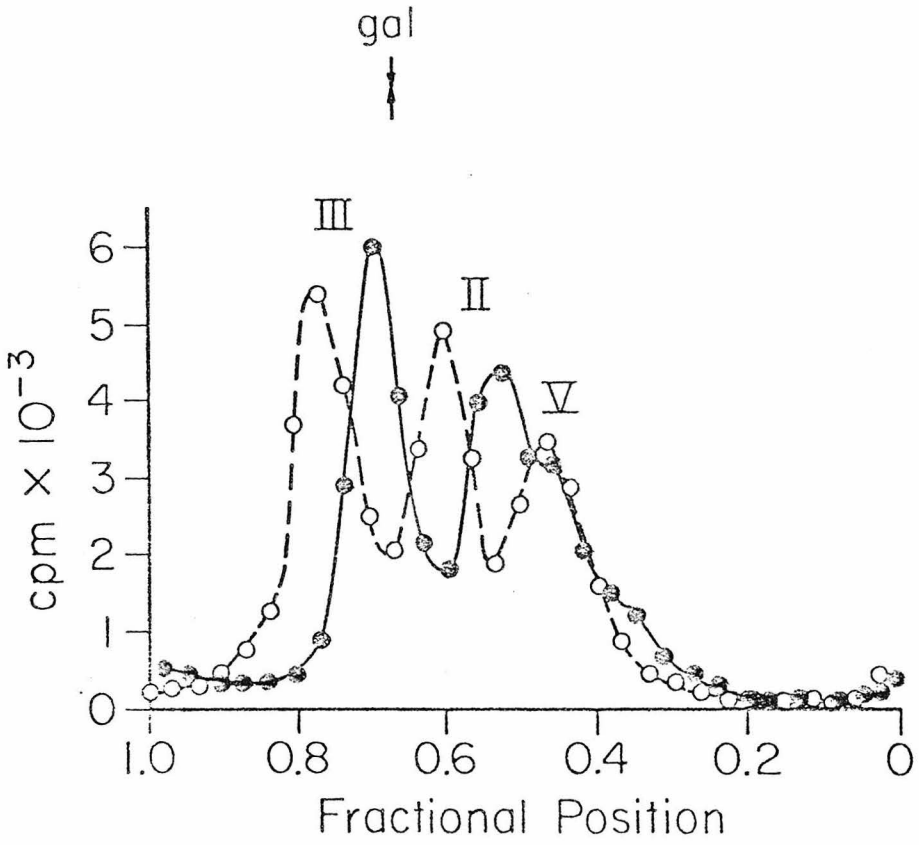
gal
III
II
V
cpm X 10^{-3}
0
1
2
3
4
5
6
1.0
0.8
0.6
0.4
0.2
0
Fractional Position

Figure 6

Legend for Table 1

Single forms of AChE or mixes of roughly equivalent amounts of several forms were incubated with or without collagenase, as in Figure 5. Aliquots of control and collagenase incubations were loaded onto 5.0 ml 5-20% sucrose gradients in 0.02 M borate, pH 8.8; 1 M NaCl; 0.1% Tween 80; 1 mg/ml BSA. β-Galactosidase, catalase, and alcohol dehydrogenase were included as markers in most of the gradients. A total of 33 gradients were run for part A of the table, and 21 gradients for part B.

After centrifugation, gradients were collected and assayed for AchE activity. The position of the activity peak was estimated to the nearest 0.1 fraction (independent estimates by different persons rarely differed by more than 0.1 fraction) and converted to a fractional position. Fractional positions in gradients not run for 5 hr were proportionally corrected, and the resulting values for each peak were used to calculate the mean fractional position and standard deviation reported in the table. The number of gradients used to calculate particular values is indicated in parentheses. Gradients with and without collagenase were tabulated separately, both for AChE activity peaks and for markers.

A. Collagenase incubations using Worthington (CLSPA) enzyme at 10 μg/ml (nominally 400 u/mg) for 1-25 hr. The

lower portion includes data from one set of gradients with no NaCl, run for 4 hr (fractional position not corrected to 5 hr).

B. Collagenase incubations using Advanced Biofactures Corp. (ABC) (Form III) enzyme. This collagenase preparation is nominally 2500 u/ml. It was used at 1-1000 u/ml for 1-12 hr.

Variation in incubation time was without effect in the range tested, except when enzyme concentrations >100 units/ml were used; in this case some degradation to form V was seen.

Table 1. Effect of Collagenase on the Sedimentation Velocity of AChE Forms

A. Worthington collagenase

1 M NaCl

	I	II	III	IV
Control	.40 ± .02	.60 ± .02	.76 ± .01	.34 ± .01
	(6)	(6)	(6)	(4)
Collagenase	.44 ± .01	.65 ± .02	.83 ± .01	.33 ± .01
	(9)	(8)	(8)	(2)

no NaCl

Control				.33
				(1)
Collagenase				.33 ± .01
				(2)

B. ABC collagenase

1 M NaCl

	I	II	III	IV
Control		.60 ± .02	.77 ± .02	
		(9)	(8)	
Collagenase		.67 ± .04	.84 ± .03	
		(15)	(15)	

Table 1. Effect of Collagenase on the Sedimentation Velocity of AChE Forms (cont.)

A. Worthington collagenase

1 M NaCl

	V	-gal	cat	ADH
Control	.52 ± .01	.68 ± .01	.48 ± .01	.33 ± .02
	(3)	(8)	(4)	(9)
Collagenase	.52 ± .01	.67 ± .01	.48 ± .02	.33 ± .01
	(4)	(9)	(6)	(12)

no NaCl

Control	.51	.69	.50	.33
	(1)	(1)	(1)	(1)
Collagenase	.50 ± .02	.68 ± .01	.50 ± 0	.33 ± 0
	(2)	(2)	(2)	(2)

B. ABC collagenase

1 M NaCl

	V	-gal
Control	.49 ± .02	.69 ± .03
	(7)	(5)
Collagenase	.49 ± .01	.67 ± .03
	(15)	(13)

Fig. 7. Effects of collagenase, phospholipase C, and trypsin on sedimentation of forms II and III. AChE form II (10 µl) and form III (7.5 µl) were mixed and diluted to 50 µl with 0.1 M Tris, pH 7.5. Ten µl aliquots were diluted into 100 µl of 0.05 M Tris, pH 7.5; 0.05 M NaCl; 0.005 M $CaCl_2$. One diluted aliquot received 10 µl of 0.1 M Tris and served as a control. Three other aliquots received either 10 µl of collagenase (Worthington, 100 µg/ml), 10 µl of phospholipase C (100 µg/ml) or 10 µl of trypsin (10 µg/ml), all freshly prepared. After 3 hr incubation at room temperature 10 µl aliquots of each were loaded onto 5 ml, 5-20% sucrose gradients in 0.02 M borate, pH 8.8, 0.1% Tween 80 and 1 mg/ml BSA, and β-galactosidase was added as a marker (↓). 1 M NaCl was included in the gradients of set A but not in those of set B. Set A gradients were centrifuged at 65,000 rpm for 4.5 hr, set B for 3.0 hr. Five-drop fractions were collected. Ten-µl portions from the first 20 fractions of each gradient were assayed for 30 min with 2×10^{-3} M ACh. Complete conversion yielded 113.7×10^3 cpm; the blank was 2.2×10^3 cpm, except that for the gradients after trypsin treatment, complete conversion yielded 22.7×10^3 cpm with a blank of 1.4×10^3 cpm.

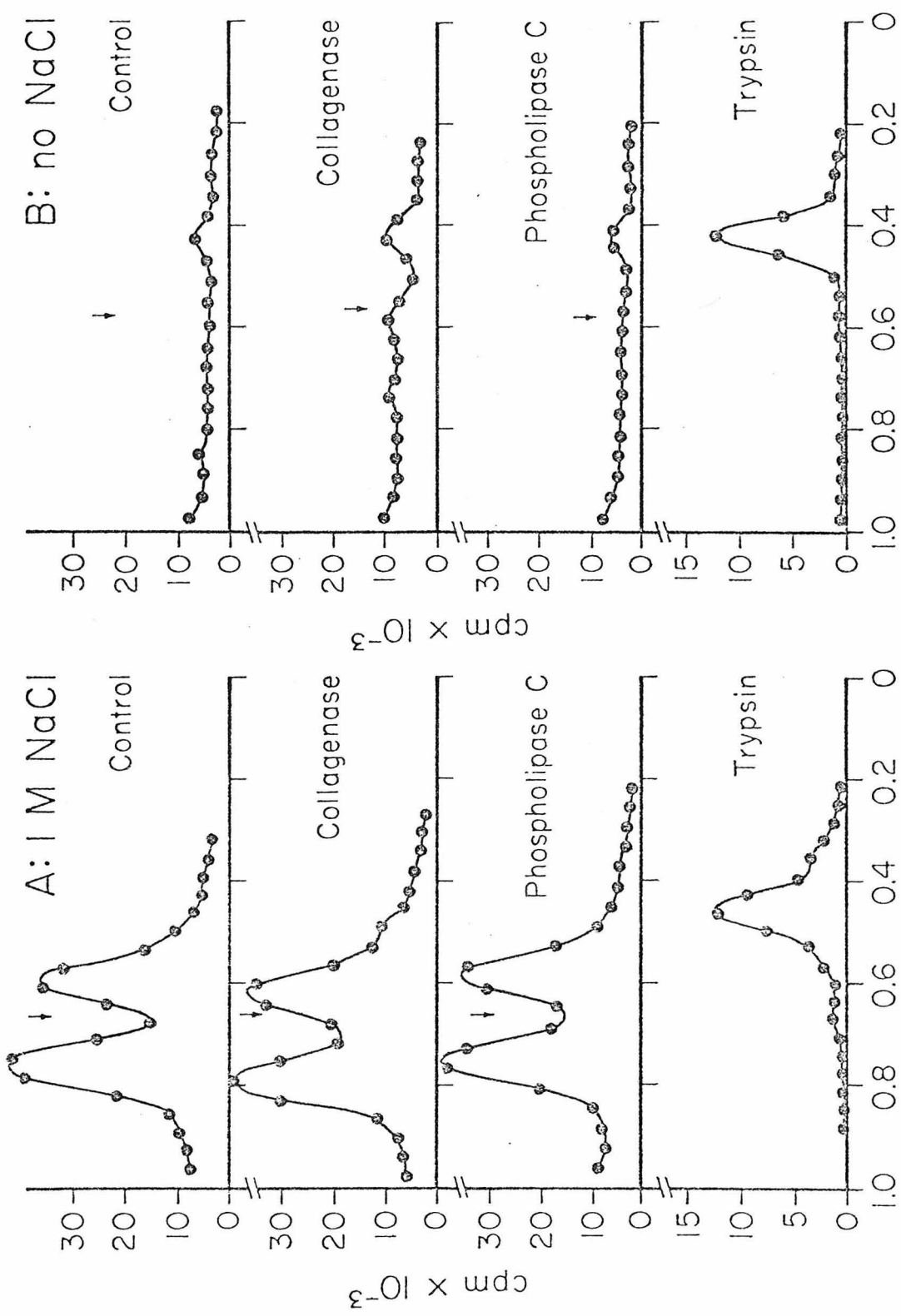
A: 1 M NaCl
B: no NaCl
Control
Collagenase
Phospholipase C
Trypsin
cpm x 10⁻³
Fractional Position
0
0.2
0.4
0.6
0.8
1.0

Figure 7

velocities than the rest (compare with marker) suggesting that they may have been more extensively modified.

The same collagenase effects were seen over a wide range of collagenase concentrations (from 0.04 u/ml to 40 u/ml), but not with other proteases or phospholipase C (Figure 7). However at very high collagenase concentrations or with incubations at higher temperatures, a conversion of forms II and III to form V was observed. This conversion is similar to that reported earlier for collagenase (Dudai & Silman, 1973, 1974a) and for trypsin and chymotrypsin (Massoulie & Ringer, 1969; Massoulie et al., 1970). Whether it represents the effect of contaminating nonspecific proteases or a more complete digestion by collagenase is unclear. (From experiments with trypsin under identical incubation conditions, we calculate that contamination of the collagenase preparation with as little as 0.01% trypsin or related nonspecific protease could produce the observed conversion to form V.)

Previous studies of native acetylcholinesterase forms II and III, using SDS-polyacrylamide gels, have shown one major subunit of ~82,000 molecular weight; after prolonged autolysis or deliberate trypsin digestion to produce form V, this subunit shows varying extents of cleavage, yielding a ~59,000 molecular weight fragment with a DFP labeled site and a ~25,000 molecular weight

fragment without (Dudai & Silman, 1974b). The results of Figure 8 confirm and extend these observations. Our preparations of the native forms II and III contain primarily the native subunit, but also significant amounts of the cleaved, active-site-containing fragment, indicating that cleavage need not alter the sedimentation properties of these native forms. In addition, virtually the same ratio of native subunit to cleaved fragment is seen after collagenase or trypsin digestions which do produce sedimentation changes, indicating that these enzymes do not (under these conditions) attack the active subunit. Instead, they must attack a different component of the native forms, one which is covalently unlinked to the active subunit but is particularly important in determining sedimentation properties.

Discussion

The ammonium sulfate fractionation procedure described above has served to separate Electrophorus electricus acetylcholinesterases into two discrete classes with distinct properties. Members of the first class (forms I, II, and III) are insoluble in low ionic strength and sediment slightly more rapidly after collagenase treatment. Previous studies indicate that these forms

Fig. 8. Effects of collagenase and trypsin on SDS-polyacrylamide gel electrophoresis of form III. AChE form III was incubated with 2 x 10^{-5} M [^{3}H]-DFP at room temperature for 20 min. Unbound DFP was removed by gel filtration on Sephadex G50. One hundred-µl aliquots of the labeled AChE were incubated with digestive enzymes. The control received 10 µl of 0.1 M Tris; the collagenase incubation received 10 µl of 100 µg/ml collagenase (Worthington CLSPA); the trypsin incubation received 10 µl of 1 µg/ml trypsin. After incubation at room temperature for 2 hrs. 20 µl aliquots were boiled in SDS and dithiothreitol. Twenty µl aliquots of the denatured, reduced enzyme were loaded onto a polyacrylamide gel and electrophoresed. After fixation and staining for protein, the gel was sliced with an array of razor blades and the distribution of [^{3}H] was determined by liquid scintillation counting. Cpm of [^{3}H] are plotted against slice number. The position of protein stain corresponding to marker BSA (66,600 dalton) is indicated with an arrow.

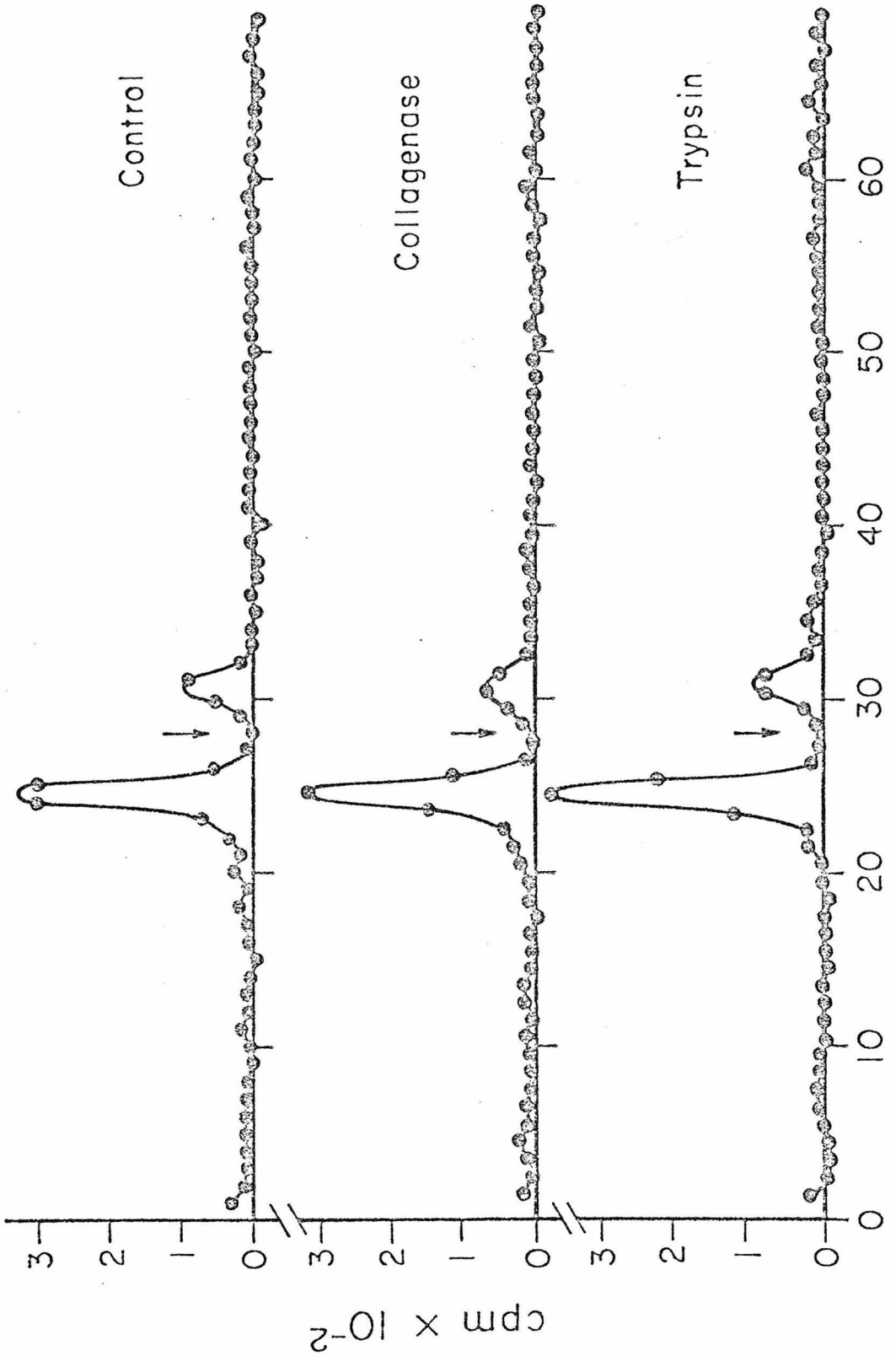
Control
Collagenase
Trypsin
cpm x 10-2
Slice Number
0
1
2
3
10
20
30
40
50
60

Figure 8

possess a 500 Å tail (Dudai et al., 1973; Rieger et al., 1973). Members of the second class (forms IV and V) are soluble in low ionic strength and are unaffected by collagenase treatment. Previous studies indicate that these forms lack a tail (Dudai et al., 1973; Rieger et al., 1973). Because forms I (9 S) and IV (7.5 S) are similar in size, they have been unresolved in many previous studies, and the reported properties of the "peak A" in which they have been jointly included have sometimes been confusing (Massoulie & Rieger, 1969; Dudai & Silman, 1974a); the ammonium sulfate procedure provides a convenient method for separating these forms. Interestingly, the order of elution in the ammonium sulfate fractionation procedure suggests that the 500 Å tail, in addition to causing precipitation at low ionic strength, also promotes "salting out" at high ammonium sulfate concentrations.

The observed effects of collagenase on forms I, II, and III differ from those previously reported. In previous studies, using high collagenase concentrations, the effects of collagenase treatment have varied; in one case the effects resembled those of nonspecific protease digestion in that the native forms II and III were partially or wholly converted to a form with the low-ionic-strength solubility and the sedimentation constant of the globular form V (Dudai & Silman, 1973, 1974a).

In a second case, collagenase was reported to make the native forms low-ionic-strength soluble without markedly altering their sedimentation properties (Rieger, Bon & Massoulie, 1973). We confirm the occurrence of the former effect at high collagenase concentration; however, we do not see either effect at low concentrations, which nonetheless produce sedimentation shifts of forms I, II, and III. Accordingly we conclude that the sedimentation shifts are more likely to be the result of specific collagenase action. Only one previous report of similar sedimentation shifts is known to us; this occurred after treatment with a "phospholipase C" preparation from Clostridium perfringens (Rieger, Bon & Massoulie, 1973). However, in the same report a Bacillus cereus phospholipase C preparation was shown to have no effect, and we also find a Clostridium welchii phospholipase C preparation to be ineffective. Noting that Clostridium perfringens is a rich source of collagenase, we prefer to believe that the previously reported shifts with the C. perfringens preparation may have been due to low level collagenase contamination.

The direction of the collagenase-induced shifts is consistent with the notion that collagenase is removing part of the 500 Å tail. From the relative sedimentation rates of form I (9 S, one tetramer plus tail) and form V (12 S, one tetramer only), it appears that the tail

significantly slows sedimentation and that its removal should then generate a sedimentation rate increase. However, the removal does not seem, under these collagenase treatment conditions, to be complete, since (a) the sedimentation rate of collagenase-modified form I (10 S) is still not as high as that of form V, (b) most of the collagenase-modified molecules remain low-ionic-strength insoluble, suggesting that some fragment of the tail is retained, and (c) a small fraction of the collagenase-modified molecules jointly acquire low-ionic-strength solubility and an even higher sedimentation rate, as if they had been deprived of an additional portion of the tail in a second, slower collagenase-dependent step. The use of electron microscopy to check the inference that collagenase removes the tail and to determine the extent of tail removal should prove interesting, and together with chemical studies of normal and collagenase-modified forms should serve to determine whether the tail is indeed composed of collagen.

Whatever the nature of the tail, it is most probably made of subunits which are not covalently linked to the active subunits. Whether this might be accomplished by post-synthesis, non-covalent association between the two subunit types, or by cleavage to produce the two subunits types from a larger precursor, is uncertain.

However, the former alternative makes it easier to understand the association of variable numbers of active subunit tetramers with a tail, and is particularly appealing if the tail is indeed a collagen-like component which serves to fix the enzyme to an extracellular, basement membrane site.

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III Separation and Characterization of Multiple Forms of Cholinesterase Activity from the Nematode *Caenorhabditis elegans*

Abstract

The nematode Caenorhabditis elegans has four forms of cholinesterase activity separable by velocity sedimentation in sucrose gradients: form I (5 S), form II (7 S), form III (11 S) and form IV (13 S). Most of form I was released by repeated extraction with 0.05 M borate pH 8.8. A portion of forms II, III and IV was released with buffer extraction alone, but a substantial amount of the larger forms was released only after treatment with the anionic detergent sodium deoxycholate (DOC). Forms I and II as a pair and forms III and IV as another pair share similar kinetic properties (i.e. Km, acylthiocholine substrate specificity, inhibitor specificity). Form II was more stable to thermal inactivation at 45°C than form I whereas forms III and IV were only slightly inactivated at 45°C and showed an identical timecourse of inactivation at 45°C and at 56°C. Forms I and II were inactivated by 0.5% DOC whereas forms III and IV were more stable in DOC but were suppressed by 1% Tween 80.

Introduction

Fractionation of solubilized cholinesterase activity from electric organs (Massoulie & Rieger, 1969; Dudai & Silman, 1974), from muscle (Hall, 1973; Wilson, Mettler & Asmundson, 1969), from vertebrate brain (Wenthold, Mahler & Moore, 1974; Ho & Ellman, 1969; Lim, Davis & Agranoff, 1971; Chan, et al., 1972) and from a variety of invertebrates (Haites, Don & Masters, 1972; Hildebrand, Townsel & Kravitz, 1974; Dudai, in press) has consistently revealed multiple forms of different sizes. Studies of the kinetic properties of the separated forms has demonstrated differences in Km (Miller, et al., 1973) in substrate inhibition (Hall, 1973) and in inhibitor sensitivity (Chan, et al., 1972) but the overall impression is of close similarity of kinetic properties (Massoulie & Rieger, 1969; Hall, 1973; Chan, et al., 1972). This suggests that the size differences result from specific associations of a common active-site-containing subunit with other components, either self (forming multimers) or non-self. In contrast, the biochemical characterization of cholinesterases in vertebrates has demonstrated such striking kinetic differences between ("true") acetylcholinesterase (EC 3.1.1.7) and ("pseudo") cholinesterase (EC 3.1.1.8) that the two activities are treated as separate enzymes and a variety of inhibitors and substrates

are routinely used to allow selective determinations of either enzyme without requiring prior separation (Bayliss & Todrick, 1956; Augustinsson, 1963).

Preliminary studies of the nematode Caenorhabditis elegans (R.L. Russell, unpublished) had revealed multiple forms of ChE activity separable by velocity sedimentation. In the hope of applying genetic analysis (for which this animal is well suited (Dougherty & Calhoun, 1948; Nigon, 1949; Brenner, 1974)) to the problem of the inter-relationship of the multiple forms, we have further characterized the separable forms of C. elegans cholinesterase activity. This part then, describes the solubilization and properties (Kms, substrate and inhibitor specificities, thermal inactivation and detergent effects) of four separable forms of nematode cholinesterase.

Materials

The detergents Tween 80 and sodium deoxycholate and the proteins bovine serum albumin (BSA, Fraction V), E. coli β-galactosidase, bovine catalase and electric eel acetylcholinesterase were from Sigma Chemical Co., St. Louis, Mo.

Renografin (3,5-diacetamido-2,4,6,-triiodobenzoic acid methylglucamine salt) from Squibb, Inc., Princeton N.J. was obtained from a local source. Acetylcholine

iodide, eserine sulphate, neostigmine bromide ((m-hydroxy-phenyl) trimethylammonium bromide dimethylcarbamate), isoOMPA (tetraisopropylpyrophosphoramide), DFP (diisopropylfluoro-phosphate), acetylthiocholine iodide and butyrylthiocholine iodide were from Sigma. Temik (2-methyl-2-(methylthio) propionaldehyde O-(methylcarbamoyl) oxime) was from Union Carbide Corp., Salinas, Ca. Tensilon (ethyl-m-hydroxy-phenyl)dimethylammonium bromide) was from Hoffman-LaRoche, Inc., Nutley, N.J. BW284C51 (1:5-bis-(4-allyldimethylammon-iumphenyl) pentane-3-one diiodide) was from Wellcome Research Laboratories, Beckenham, England. TEA (tetraethylammonium chloride) and hemicolinium-3 (2,2'-(4,4'-biphenylene)-bis (2-hydroxy-4,4'-dimethylmorpholinium bromide)) were from Eastman Kodak Co., Rochester, N.Y. Propionyl thiocholine iodide was from K & K Laboratories Inc., Plainview, N.Y. Paraoxon was a gift from the Department of Nematology, University of Calif., Riverside. Acetyl-β-methyl-thiocholine iodide and propionyl-β-methyl-thiocholine iodide were from U.S. Biochemical Corp., Cleveland, Ohio. 3,3'-Dithio (6-nitrobenzoic acid) (DTNB) was from Eastman. [^{3}H]-acetylcholine was TRA277 from Amersham-Searle Corp., Arlington Heights, Ill or NET-113 from New England Nuclear, Boston, Mass. Aquacide II was from Calbiochem, La Jolla, Ca. Sepharose 6B was from Pharmacia Chemicals, Inc., Piscataway, N.J. Other chemicals were standard reagent grade. Borate buffers were dilutions of 0.1 M $Na_2B_4O_7$

adjusted to pH 8.8 with HCl.

Methods

Nematodes (Caenorhabditis elegans, Bristol variety, N2 strain) were maintained in the laboratory on agar surfaces (NGMM media - Table 1) seeded with a lawn of Escherichia coli (OP-50 strain) (Brenner, 1974). The larger number of animals used for biochemical studies were grown in liquid cultures (Figure 1). For food, E. coli (NA-22 strain) was grown to stationary phase at 23°C on M9 media with 0.8% glucose as a carbon source (Table 1). The bacteria were collected by centrifugation and resuspended in S media (Table 1) at up to 30 gm/l. An inoculum of nematodes was added and the culture was aerated with several spargers (for 10 l in a 5 gallon carboy) or by the action of a reciprocal shaker (for 500 ml in a 2 l Erlenmeyer flask). (The shaker has a throw of about 4 inches and the frequency was adjusted to about 2 cycles/second to produce a sloshing of the media which was sufficient for rapid growth.) Relatively large inocula (0.5-5% of the final yield) were used to produce rapid growth yielding healthier nematodes and avoiding the danger of contamination. Carboy cultures were often seeded with the nematodes harvested from cultures grown on the shaker.

Table 1. Media

NGMM

for 4 l

agar 68 gm
NaCl 12 gm
peptone 10 gm
H_2O to 4 l

autoclave, cool
add, presterilized
1. 10 mg/ml cholesterol in ethanol 4 ml
2. 0.5 M $CaCl_2$ 8 ml
3. 1.0 M $MgSO_4$ 4 ml
4. 1.0 M (K)PO_4 = 100 ml (pH 6.0)
   35.6 gm K_2MPO_4
   108.3 gm KH_2PO_4
   H_2O to 1 l

M9

for 1 l
NaCl 5 gm
NH_4Cl 1 gm
Na_2HPO_4 6 gm
KH_2PO_4 3 gm
$MgSO_4$ 0.1 gm
H_2O to 900 ml

autoclave, cool
add, presterilized
1. 8% (w/v) glucose 100 ml

S media

for 1 l

K_2HPO_4 1 gm
KH_2PO_4 6 gm
NaCl 5.85 gm
*10 mg/ml sitosterol in Tween 80 1 ml
H_2O to 1 l
autoclave, cool
add, presterilized
1. Trace Metals 10 ml
   0.69 gm $FeSO_4 \cdot 7H_2O$
   0.20 gm $MnCl_2 \cdot 4H_2O$
   0.29 gm $ZnCl_2 \cdot 7H_2O$
   1.86 gm Na_2EDTA
   H_2O to 1 l
2. 1 M (K)citrate 10 ml (pH6.0)
   20.0 gm citric acid$\cdot H_2O$
   293.5 gm $(K)_3$citrate$\cdot H_2O$
   H_2O to 1 l
3. 1 M $MgSO_4$ 3 ml
4. 0.5 M $CaCl_2$ 6 ml

*or use 10 mg/ml cholesterol in ethanol (add after autoclaving) for bubbling cultures

Fig. 1. Growth of Caenorhabditis elegans in liquid culture.

A culture of E. coli was grown to stationary phase at room temperature and the bacteria were collected by centrifugation and resuspended in half the volume of S media. The nematodes (N2) from several 9 cm petri plates were added to 500 ml of resuspended bacteria in a 2 l Erlenmeyer flask and incubated at 20°C on a reciprocal shaker. Aliquots were removed for counting of bacteria (Petroff-Hauser slide) and of nematodes (Scott slide). During exponential growth, the nematode doubling time was about 14 hours.

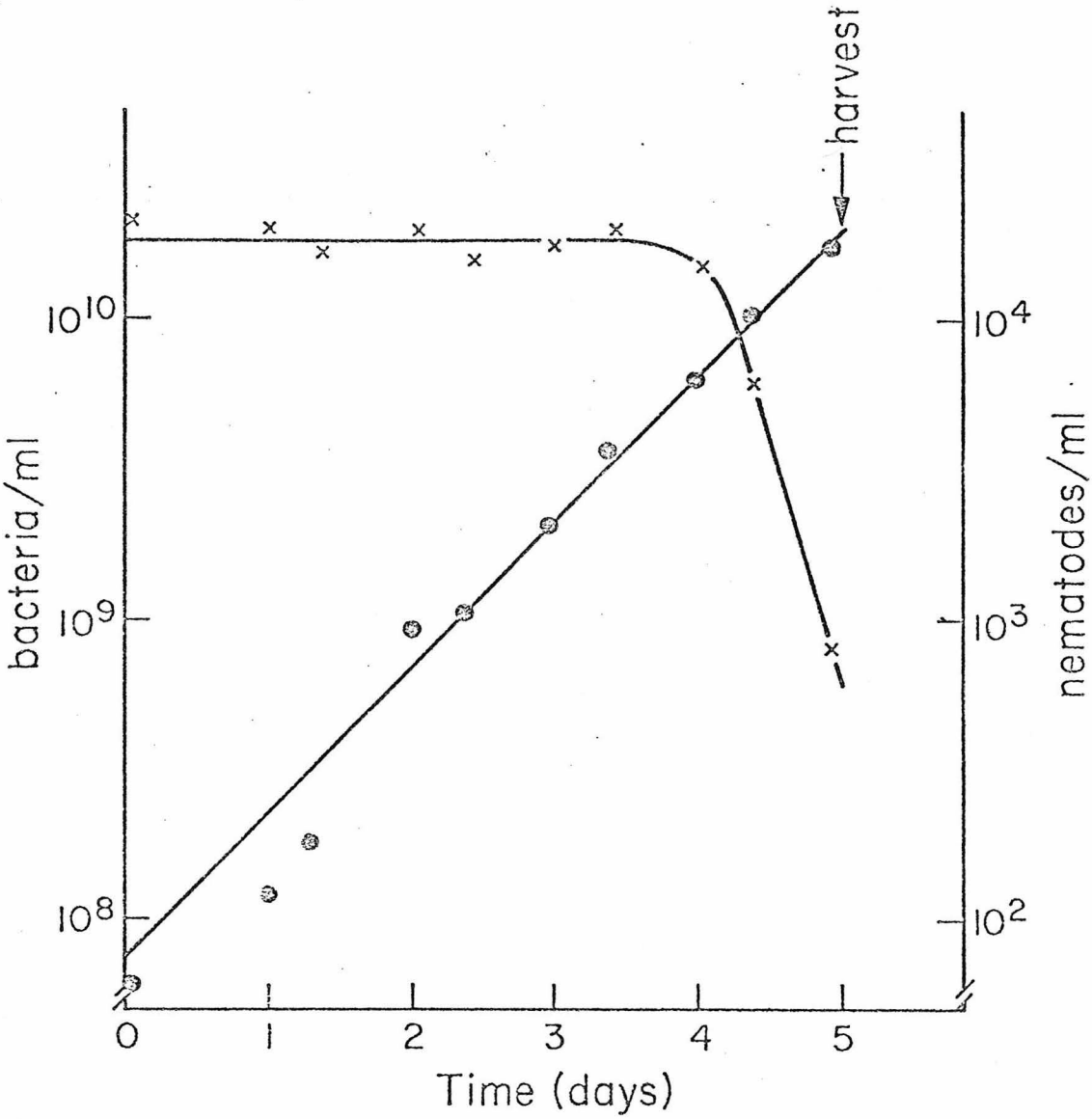
harvest
bacteria/ml
nematodes/ml
10^{10}
10^{9}
10^{8}
10^{4}
10^{3}
10^{2}
0
1
2
3
4
5
Time (days)

Figure 1

Cultures were harvested by low speed centrifugation or by settling at 4°C. After several washes with cold distilled water, the nematodes were separated from debris and crystalline material which forms in the cultures by flotation in 35% sucrose (Sulston & Brenner, 1974). After further washes to remove the sucrose, the animals were pelleted in a Sorvall centrifuge and then used immediately or frozen in liquid nitrogen and stored either under liquid nitrogen or at -20°C.

Homogenization was by freeze-powdering in liquid nitrogen. Usually, a small amount of water was added to allow the pelleted animals to be pipetted and they were then added dropwise to liquid nitrogen in a cold mortar. The frozen droplets were ground to a fine powder with a cold pestle and the powder was removed with a spatula and thawed. Subsequent fractionation was performed at 0-4°C. Pellets were resuspended by re-freeze-powdering or with a Dounce glass homogenizer.

The multiple molecular forms of cholinesterase were displayed by zone velocity sedimentation through sucrose gradients (Martin & Ames, 1961) using either a SW 27 or a SW 65 rotor in a Beckman L2-65B ultracentrifuge. SW 27 gradients were collected by lowering a thin capillary pipette to the bottom of the tube and pumping the contents out with a peristaltic pump. SW 65 gradients were collected by drop after puncturing the bottom of the tube

with a rigidly held needle. Catalase (11.3 S) and β-galactosidase (15.9 S), included in some gradients as markers, were assayed by spectrophotometric assays (Massoulie & Rieger, 1969; Baudhuin, et al., 1964).

For biochemical characterization, and to permit re-running of separated forms, the appropriate fractions of sucrose gradients were dialyzed against 0.05 M borate, pH 8.8 buffer and concentrated with Aquacide II. Sucrose was removed from larger fractions by pressure filtration in a Diaflo apparatus using a PM30 membrane, then further concentrated in a dialysis bag with Aquacide II. Separated, concentrated forms were further dialyzed against buffer then stored at -20°C.

Assays for cholinesterase were with a radiometric liquid extraction assay (Johnson & Russell, 1975). Assays included, in 100 µl, 0.025 M (K)$PO_4^=$, pH 7.0 and 0.02-0.1 µcurie [^{3}H]-acetylcholine. Cold ACh was usually included to give 5.0 x 10^{-5} M ACh. Assays were started by the addition of enzyme or substrate and stopped with 100 µl of 1 M $ClCH_2COOH$, 0.5 M NaOH, 2 M NaCl; four mls of scintillation fluid (0.5% PPO, 0.03% POPOP in toulene-10% n-butanol) was added, the reaction-scintillation vial was capped, vigorously shaken and counted. The labelled product ([^{3}H]-acetic acid) extracts into the toluene phase and is selectively counted. Each set of assays included several blanks and complete conversions (effected with

10 µl of 100u/ml electric eel AChE) as controls.

The Km for acetylcholine was determined by assays containing variable amounts of cold ACh and a constant amount of [^{3}H]-ACh. Buffer and substrates were premixed and 100 µl was used for duplicate assays of each enzyme being tested and for triplicate blanks and complete conversions. Enzymes were prepared at 4°C in 0.05 M borate, 1 mg/ml BSA, at a concentration that produced about 30% conversion at the lowest concentration of ACh and were then allowed to equilibrate to room temperature for 10 minutes before beginning the assay.

Specificity toward acylthiocholine substrates was determined with the spectrophotometric assay of Ellman (Ellman, et al., 1961) modified so as to approximate the conditions of the radiometric assay. Each assay contained 0.86 ml 0.025 M (K)$PO_4^=$, pH 7.0, 0.1 ml 0.0033 M DTNB and 0.02 ml 0.05 M acylthiocholine iodide (final concentration 10^{-3} M). The assay was started by addition of enzyme in 0.02 ml. The change in OD_{412} was followed on an Acta III recording spectrophotometer with a blank (no enzyme) in the reference cell.

The effects of inhibitors were assessed by radiometric assay at 3 x 10^{-6} M ACh (no cold ACh added). Inhibitors were prepared fresh and premixed with buffer and substrate; 100 µl was used for duplicate assays of each enzyme being tested and for triplicate blanks and

complete conversions. Complete conversion was achieved with 10 µl of 2 M NaOH and neutralized with 10 µl of 1 M H_2SO_4 before stopping with the usual solutions.

Thermal inactivations were performed by diluting enzyme at least 20X-fold into assay buffer preheated to the inactivation temperature. Aliquots were removed at specific times thereafter and transferred to assay vials on ice. After all time points were taken, the vials were warmed to room temperature and the assay was started by the addition of substrate.

Results

When nematodes were homogenized by freeze-powdering in liquid nitrogen and thawed into 5 volumes of distilled water, only a small amount ($\leq$ 5%) of the cholinesterase activity measurable in the homogenate was solubilized. For preparative studies, the diluted homogenate was usually centrifuged at 20,000 rpm for 30 min and cholinesterase was extracted from the resulting pellet by resuspension in an extraction buffer. Treatment of the homogenate for 2 hours at 4°C with 0.05 M borate, pH 8.8 released 47% of the measurable activity to a low speed supernatant. Analysis of this supernatant by velocity sedimentation (Figure 2) revealed two peaks of solubilized activity at 5 S and at 12 S. In addition, a substantial portion of the activity was not solubilized and floated on a cushion

Fig. 2. Fractionation of Cholinesterase by velocity sedimentation.

Fresh worm homogenate was diluted into 10 volumes of 0.05 M borate, pH 8.8. After two hours stirring at $4^{o}C$, a 1500 g, 5 min supernatant was prepared (containing 47% of the activity of the homogenate) and 1 ml was loaded unto 34 ml 5-20% sucrose gradients (same buffer) with 4 ml cushions of Renografin. The gradient was centrifuged for 25 hours at 27,000 rpm. Fractions were collected and 10 µl of each was assayed for 90 min with 5×10^{-5} M ACh. The blank of [^{3}H]-ACh was 0.3×10^{3} cpm; complete conversion was 18.0×10^{3} cpm. The position of β-galactosidase from E. coli (15.9 S) is indicated by the arrow.

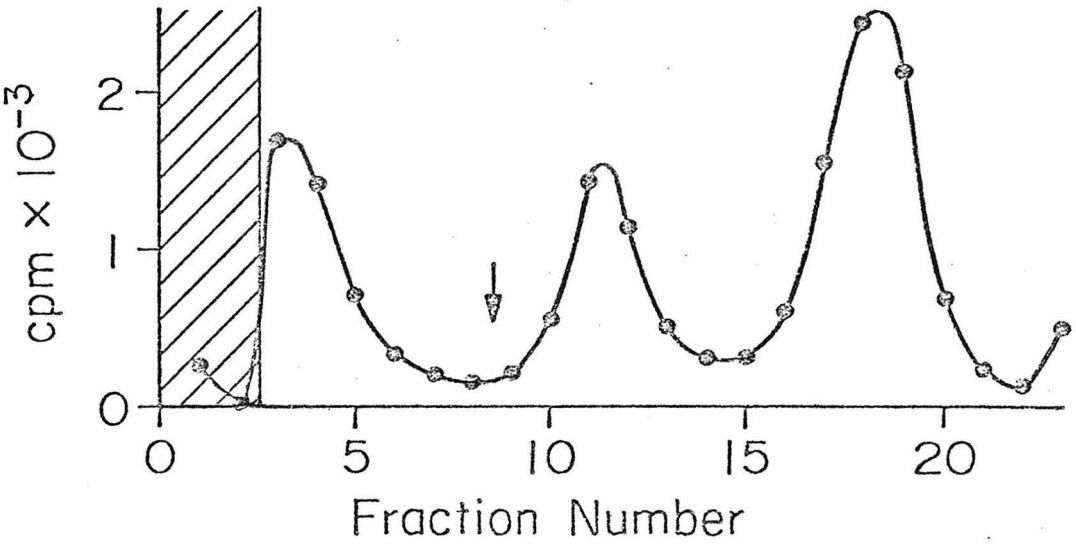

Figure 2

of high density Renografin. (When a cushion was not used, this fraction pelleted and was not recovered.) Extraction with high ionic strength solutions (0.05 M borate, pH 8.8; 1 M NaCl) did not appreciably enhance the amount of activity released and no additional sedimentation peaks were revealed (Figure 3). Repeated extraction of high speed pellets with 0.05 M borate, pH 8.8, over several days following homogenization (Figure 4A) released about half of their total activity to supernatants. Much of the released activity in the first few supernatants sedimented at about 5 S. The 5 S peak decreased in the later extractions to reveal an additional peak at about 7 S. In addition, all of the supernatants contained material sedimenting as a broad peak from 11 S to 13 S.

Various treatments were tried in attempts to release the activity which remained in the pellet. Only treatment with the anionic detergent sodium deoxycholate (DOC) released most of the activity (> 85% in two washes). The activity extracted from the pellet by DOC sedimented as a 7 S peak and as a broad 11-13 S peak (Figure 4B). Only small amounts of activity sedimented at the 5 S position.

When gradient fractions were dialyzed and refractionated by velocity sedimentation (Figures 5 and 6) sharp peaks were observed at four separate positions corresponding to four separable forms: form I (5.3 ± 0.3 S), form II

Fig. 3. Velocity sedimentation after extraction with 1 M NaCl.

Fresh worm homogenate was diluted six fold into 0.05 M borate, pH 8.8; 1 M NaCl. After 2 hours stirring at $4^{o}C$, a 1500 g, 5 min supernatant was prepared and 1 ml was loaded unto 38 ml 5-20% sucrose gradients. The gradients were centrifuged at 27,000 rpm for 16 hours. Fractions were collected and 10 µl of each was assayed with 5.0×10^{-5} M ACh for 45 min. The blank was 0.2×10^{3} cpm; complete conversion was 17.6×10^{3} cpm.

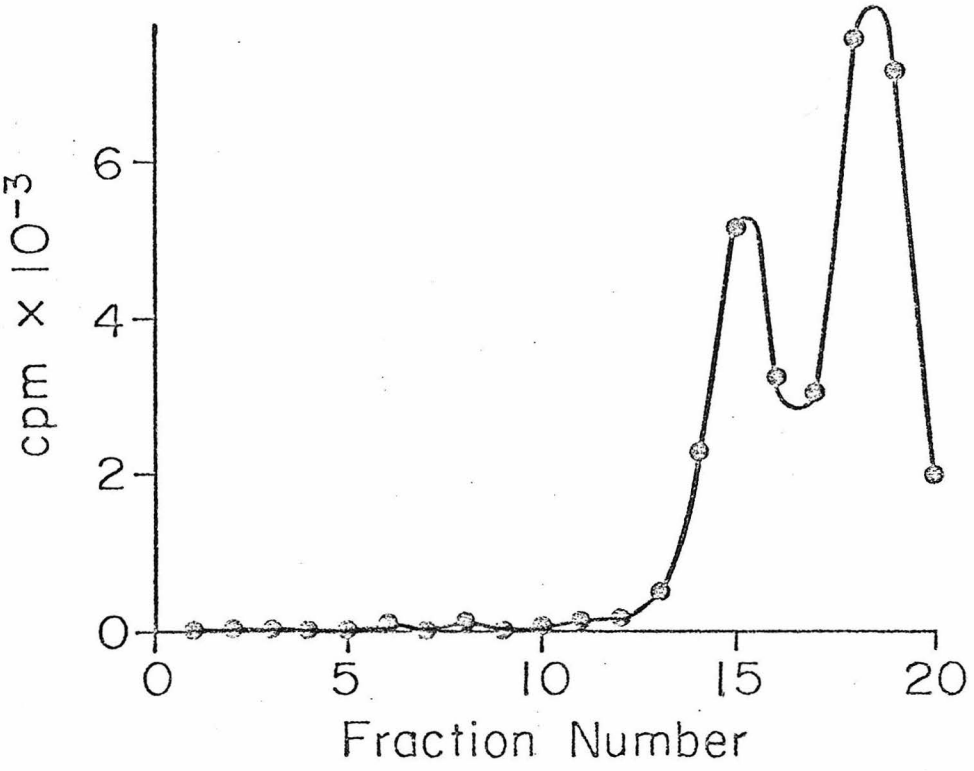

Figure 3

Fig. 4. Successive extractions with 0.05 M borate, pH 8.8.

About 20 gm of fresh nematodes (N2) were homogenized by freeze powdering. Without dilution, a 20,000 rpm, 30 min supernatant was prepared (yield 6 ml-used for studies of soluble enzymes). The pellet was resuspended in distilled water and sedimented at 20,000 rpm for 30 min. The two S20K supernatants were respun at 40,000 rpm for 30 min to give S40K-1 and S40K-2. The combined pellet was repeatedly resuspended by Dounce homogenizer in 10-20 ml of 0.05 M borate, pH 8.8 as a series of nine 40,000 rpm, 30 min supernatants (S40K-3 thru S40K-11) were prepared over 4 days. A. Total S40K supernatant activity (1 u = 1 mole ACh hydrolyzed/min at 5×10^{-5} M ACh) is plotted against the time of supernatant preparation. The dashed lines above S40K-5 and S40K-9 represent activity in the resuspended pellet.

B. Velocity sedimentation of the S40K supernatants at 65,000 rpm for 6.5 hrs (5 ml 5-20% sucrose gradients in 0.05 M borate, pH 8.8). Slight differences in sample size, length of run and number of fractions were compensated for by plotting activity versus fractional position. Activity assays were with 5.0×10^{-5} M ACh and 10^5 cpm of [^{3}H]- ACh. The measured cpms (blank subtracted) were corrected to a 10 µl, 10 min assay and multiplied by the total volume of each S40K so that the plotted activity was

proportional to total activity of the fraction. The final gradient represents activity solubilized by treatment of P40K-9 with 0.5% DOC.

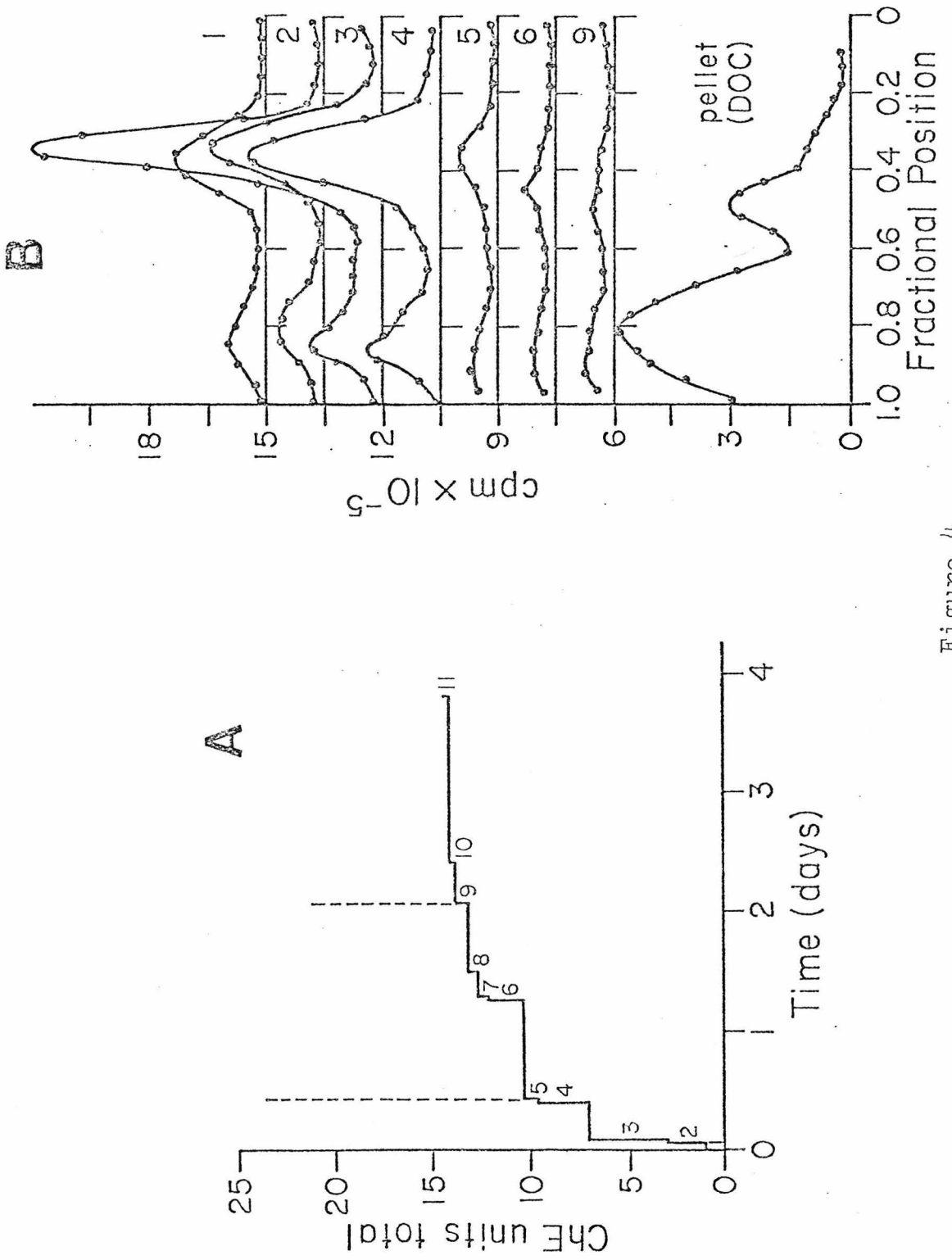
A
CHE units total
25
20
15
10
5
0
0
1
2
3
4
Time (days)
2
3
4
5
6
7
8
9
10
11
B
cpm x 10⁻⁵
18
15
12
9
6
3
0
1
2
3
4
5
6
9
pellet (DOC)
1.0
0.8
0.6
0.4
0.2
0
Fractional Position

Figure 4

Fig. 5. Velocity sedimentation of separated peaks.

The peak fractions from sucrose gradients were pooled, dialyzed and concentrated. Aliquots were refractionated by velocity sedimentation at 65,000 rpm for A. 12 hours, B. 9 hours and C. 6.5 hours. Samples were A. 5 S peak of the first pH 8.8 extract (S40K-3, Figure 4); B. 7 S peak from a fractionation of later pH 8.8 extracts (pooled S40K-5 thru S40K-9, Figure 4) and C. 11-13 S peak from the first pH 8.8 extract (S40K-3, Figure 4). The peaks are labelled I-IV representing the four separated forms shown completely separated in Figure 6. The arrow marks a comparable position in the three gradients. Assay blanks were 0.5×10^3, 0.6×10^3, and 3.7×10^3 cpm and complete conversions were 18.2×10^3, 16.4×10^3 and 96.5×10^3 cpm for panels A, B and C respectively.

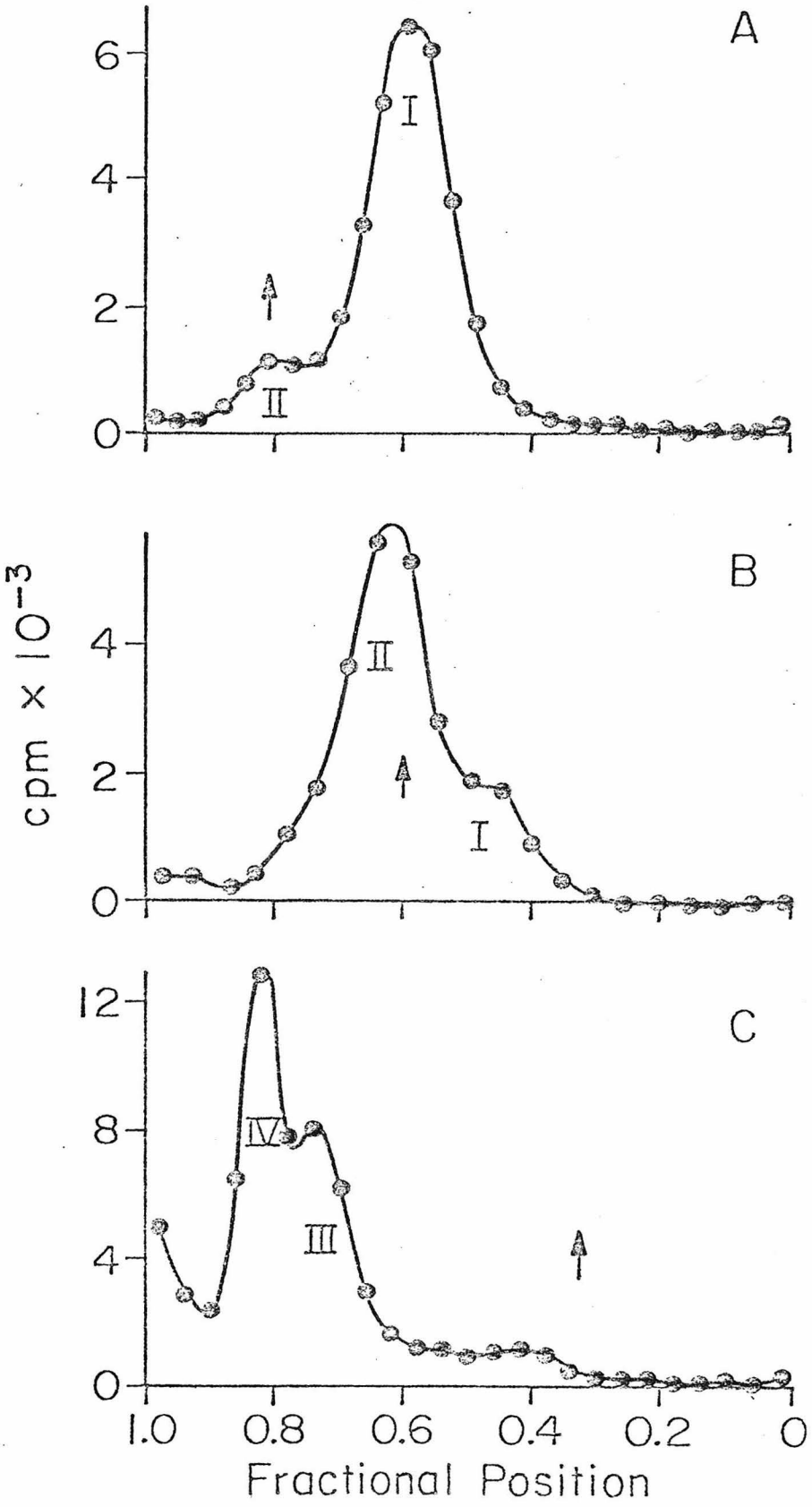
A
B
C
I
II
III
IV
6
4
2
0
12
8
cpm x 10⁻³
1.0
0.8
0.6
0.4
0.2
0
Fractional Position

Figure 5

Fig. 6. Rerun of separated forms.

A one hundred µl aliquot of separated forms I-IV were loaded unto a sucrose gradient in 0.05 M borate, pH 8.8. After sedimentation for 7 hours at 65,000 rpm, fractions were collected and assayed with 5.0×10^{-5} M ACh. The arrow indicates the position of bovine catalase (11.3 S).

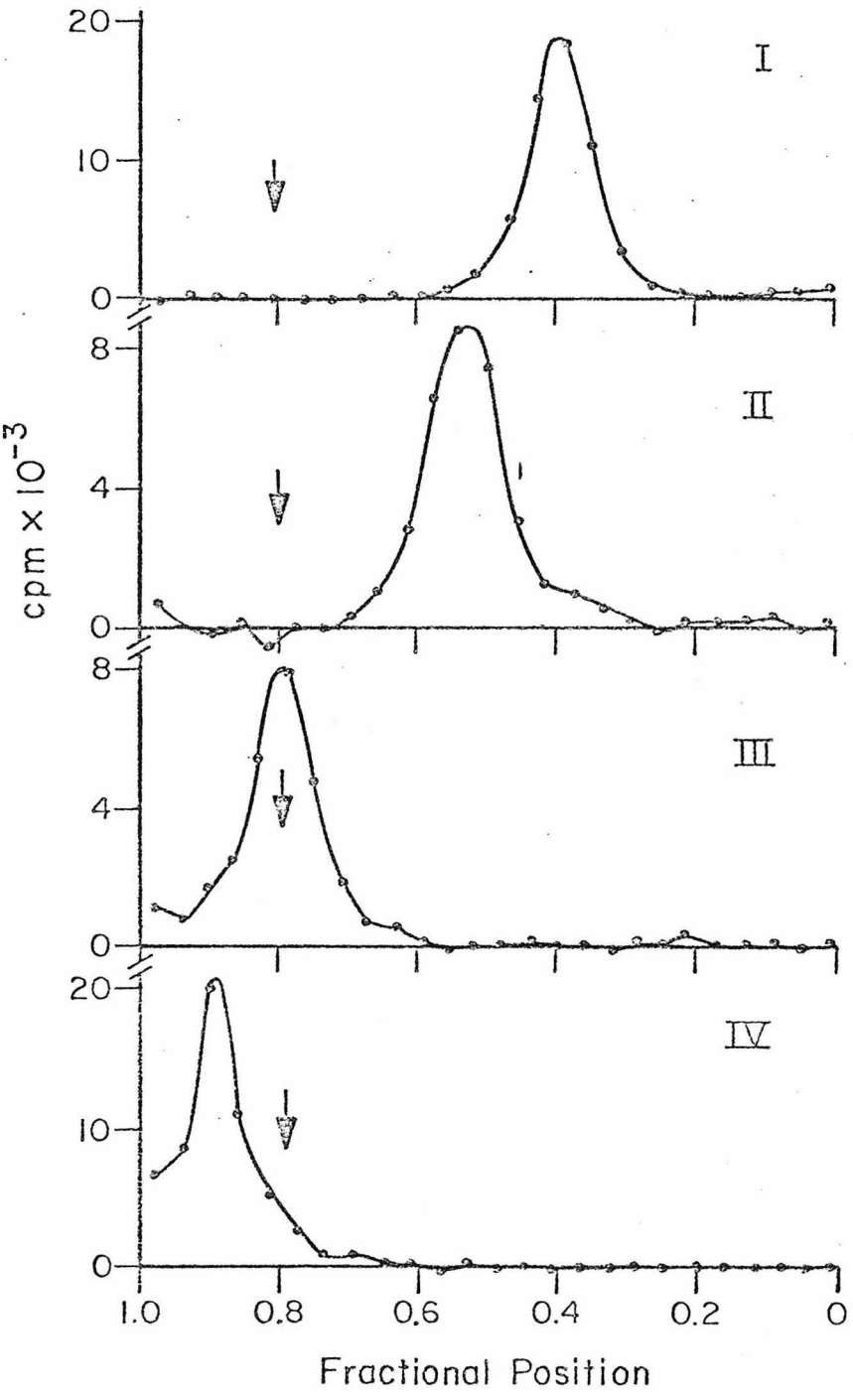
20
10
0
I
8
4
0
II
cpm x 10^{-3}
8
4
0
III
20
10
0
IV
1.0
0.8
0.6
0.4
0.2
0
Fractional Position

Figure 6

(7.1±0.4 S), form III (11.4±0.5 S) and form IV (13.0±0.6 S). A number of cholinesterases have been reported to be "elongated" based upon a comparison of velocity sedimentation and gel filtration data (Massoulie, Rieger & Bon, 1971; Dudai, in press); by comparison to a globular protein of equivalent molecular weight, elongation leads to slower sedimentation (suggesting a lower molecular weight) but earlier elution from a gel filtration column (suggesting a higher molecular weight). None of the worm cholinesterases appear to be substantially elongated by this comparison (Table 2). The molecular weights of the forms, calculated from sedimentation coefficient are: form I (82,000±5000), form II (130,000±10,000), form III (275,000±15,000) and form IV (343,000±22,000).

Since multiple forms could be produced by degradative processes during the relatively lengthy extractions, attempts were made to directly solubilize all of the cholinesterase activity of the homogenate. When aliquots of variously extracted homogenate were sedimented on gradients with Renografin cushions, it was found that a 30 minute treatment with 0.5% DOC completely eliminated activity on the cushion. Solubilization with DOC was complicated by a rapid loss of activity sedimenting at 5 S and 7 S and maximal recovery of the solubilized activity was achieved by short (1 min) treatments with 0.2% DOC

Table 2. Average Sedimentation Constants and Gel Filtration of Separated Froms

	Velocity Sedimentation		Gel Filtration	
Form	S	estimated* MW ($\times 10^{-3}$ daltons)	Kav (6B)	estimated* MW ($\times 10^{-3}$ daltons)
I	5.3 ± 0.3 (n=7)	82 ± 5	0.53	95
II	7.1 ± 0.4 (n=5)	130 ± 10	0.48	135
III	11.4 ± 0.5 (n=6)	275 ± 15	0.34	340
IV	130 ± 0.6 (n=6)	343 ± 22	0.32	390
Drosophila melanogaster	7.4 ± 0.4 (n=3)	135 ± 10	0.41	220
Electric eel (V)	12.8	305	0.32	390

Kav determined relative to blue dextran (Kav = 0.0) and tryptophane (Kav = 1.0) on a column (1 cm dia x 34 cm) of Sepharose 6B (run in 0.02 M borate, pH 8.8; 0.1% Tween 80). The marker positions were determined by continuous monitoring of the OD_{280} of the eluate with a LKB Unicord II spectrophotometer.

*Molecular weights were estimated for sedimentation from plots of literature S_w^{20} and MW data. For the gel filtration data, MW was estimated off the chart in the Pharmacia Chemical Co. pamphlet "Sepharose".

followed as quickly as possible by sedimentation into gradients without DOC and containing 0.1% Tween 80 (Figure 7). The distribution of activity in such gradients was smeared, but consistent with the presence of all of the separable forms *in vivo*. Forms I and II have been reliably recovered from reruns of DOC extract gradient fractions. The separation of the larger forms into only forms III and IV was usually less clear and there exists the possibility of additional minor heterogeneity here.

Biochemical characterization of the four separated forms is described in Figures 8 and 9 and Tables 3, 4 and 5.

Kms

Forms I and II as a class have similar Kms which are significantly different from those of forms III and IV (Figure 8). Electric eel AChE and horse serum cholinesterase had Kms of 7.0×10^{-5} M and 32.3×10^{-5} M in the same experiment. (The radiometric assay is not well suited for use at the higher concentrations of ACh necessary to assess substrate inhibition and the precise determination of the extent of substrate inhibition for all of the worm forms has not been completed. An experiment shown in Part IV (Figure 7) demonstrates the substrate inhibition of forms I and III.)

Substrate specificity

Table 3 presents the substrate specificity for a

Fig. 7. Velocity sedimentation after extraction with DOC.

One ml of frozen nematodes were freeze powdered and extracted with 0.8 ml of 0.1 M borate, pH 8.8 and 0.03 ml of 10% DOC (final 0.17% DOC) at 4^{o}C. After 2 min, a 1500 g, 1 min supernatant was prepared and 100 µl aliquots were loaded unto 5.0 ml 5-20% sucrose gradients in 0.05 M borate, pH 8.8, 0.1% Tween 80. After centrifugation (65,000 rpm, 4.5 hours), fractions were collected and 10 µl of each was assayed for 10 min with 5.0×10^{-5} M ACh. The blank was 1.1×10^{3} cpm; complete conversion was 32.0×10^{3} cpm. Alternate fractions were reassayed with 10^{-5} M eserine —o— .

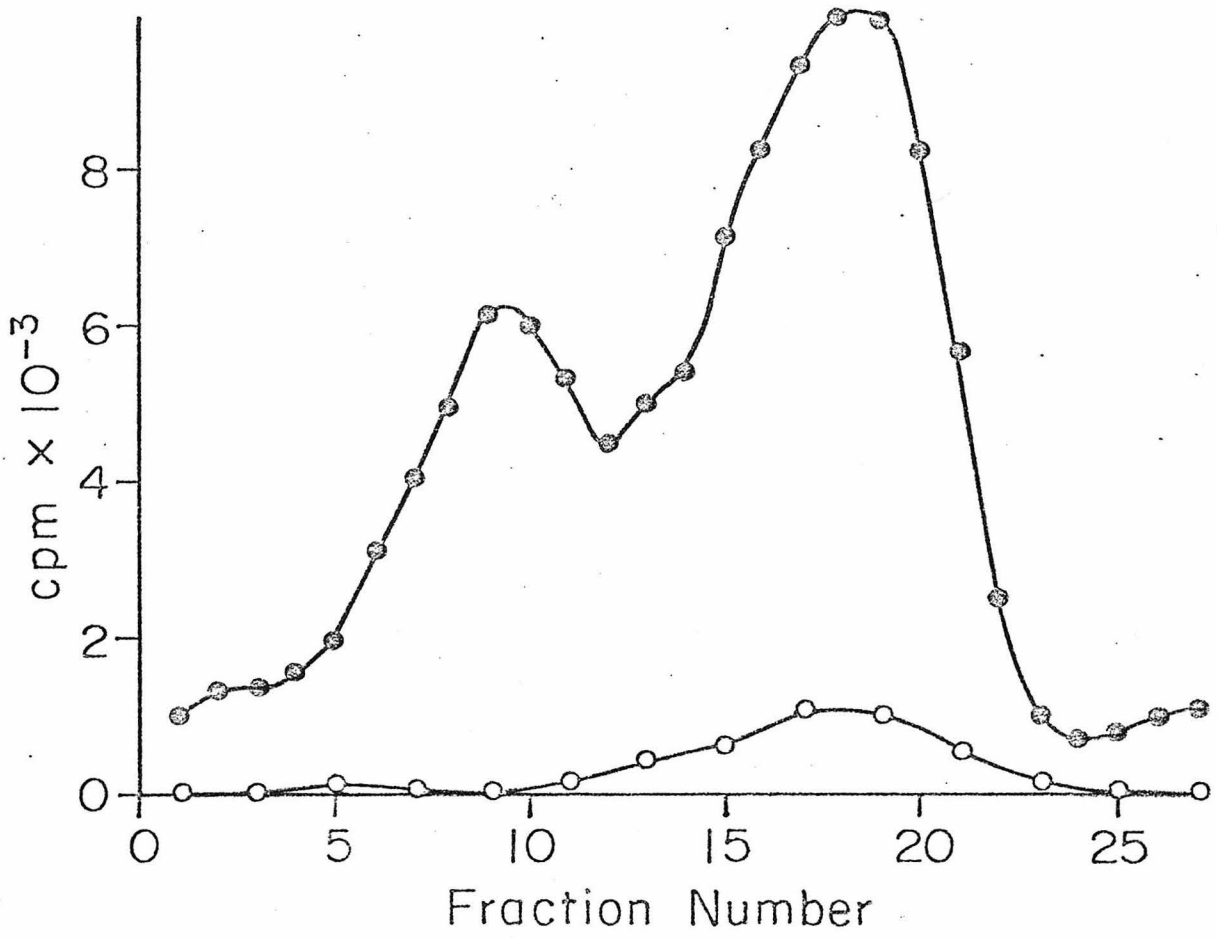

Figure 7

Fig. 8. Km for acetylcholine.

The separated forms I-IV were assayed with 10 concentrations of ACh ranging from 2×10^{-6} to 10^{-4} M. Velocity (V in u/ml) was plotted against V/S, an Eadie-Hofstee plot. The Km (-slope) was calculated with the linear regression program on an HP65 calculator. The standard deviations of the slopes were: form I $\pm$ 0.6×10^{-5}M, form II $\pm$ 0.5×10^{-5} M, form III $\pm$ 0.1×10^{-5} M, form IV $\pm$ 0.1×10^{-5} M.

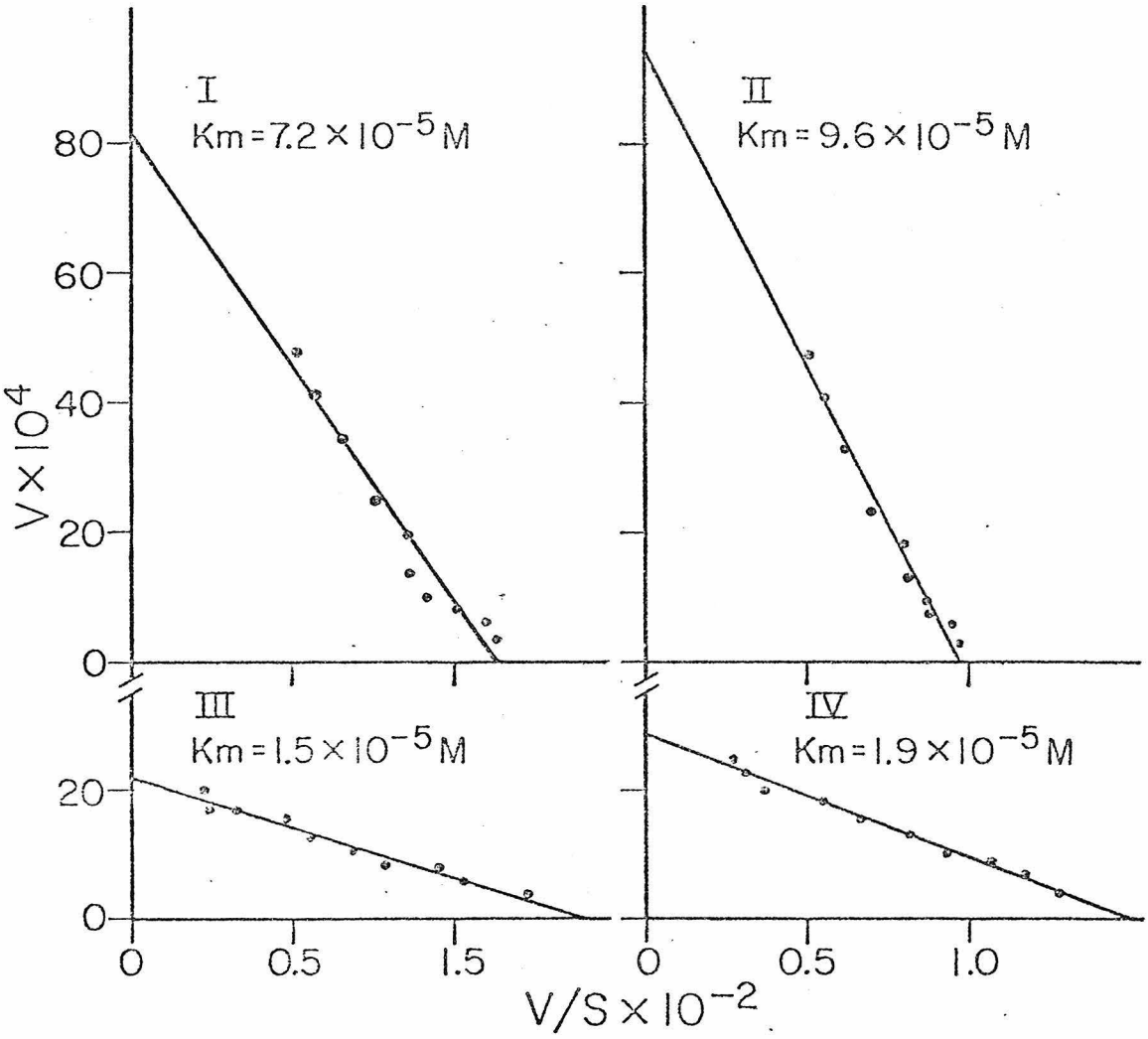
I
Km = 7.2 × 10⁻⁵ M
II
Km = 9.6 × 10⁻⁵ M
III
Km = 1.5 × 10⁻⁵ M
IV
Km = 1.9 × 10⁻⁵ M
V × 10⁴
80
60
40
20
0
20
0
0
0.5
1.5
0
0.5
1.0
V/S × 10⁻²

Figure 8

Table 3. Acyl-thiocholine Substrate Specificity of Separated Forms

Substrate	Activity of electric eel-AChE	horse serum ChE	nematode ChE forms I	II	III	IV
acetyl-thiocholine	100	100	100	100	100	100
acetyl-β-methyl-thiocholine	68	37	45	47	66	78
propionyl-thiocholine	61	189	54	59	104	103
propionyl -β-methyl thiocholine	36	92	35	34	94	102
butyrylthiocholine	0.3	181	26	25	64	71

Hydrolysis of acyl-thiocholine substrates was followed spectrophotometrically as described in methods. Substrates were at 1 mM. All of the enzymes had about the same activity with acetylthiocholine as substrate. Activity (Δ OD_{412}/min) recorded as percent of the activity measured with acetylthiocholine.

number of acylthiocholine compounds. The "true" acetylcholinesterase from the electric eel and the "pseudo" cholinesterase from horse serum are included for comparison. All of the nematode enzymes are intermediate in specificity between the two vertebrate enzymes. Comparison of the four separable forms of worm ChE again reveals a pairing of forms I and II and of forms III and IV. In general the rate of hydrolysis with forms III and IV was less effected by lengthening the acyl group than was that of forms I and II.

Inhibitor specificity

The inhibition of electric eel AChE, horse serum ChE and the four separated forms of worm cholinesterase with nine cholinesterase inhibitors (Table 4) divided the four worm forms into the same two classes seen previously in Km and substrate specificity data. The inhibitors were chosen to include several covalent carbamate and organophosphate inhibitors as well as reversible inhibitors. Two of the inhibitors are 5×10^{-4} M TEA, commonly used to block potassium-channels ($Kd=4 \times 10^{-4}$ M, Hille, 1967), and 5×10^{-6} M hemicolinium-3, used to block choline uptake (at 2×10^{-5} M, Birks & MacIntosh, 1961); both of these compounds are effective cholinesterase inhibitors. The nematode enzymes were only 5-11% inhibited by 2×10^{-3} M isoOMPA, the specific inhibitor of "pseudo"

Table 4. Inhibitor Specificity of Separated Form

Inhibitor	% Inhibition of electric eel AChE	horse serum ChE	nematode ChE form I	II	III	IV
neostigmine (2×10^{-7}M)	78	22	25	19	73	76
temik (2×10^{-5}M)	89	71	31	20	56	59
DFP (10^{-6}M)	10	98	29	22	58	63
paraoxon (10^{-6}M)	44	87	20	22	15	15
iso-OMPA (2×10^{-3}M)	23	100	5	7	9	11
tensilon (2×10^{-6}M)	96	0	34	30	61	59
BW284C51 (2×10^{-6}M)	99	21	45	48	64	69
TEA (5×10^{-4}M)	91	26	68	66	76	75
hemicolinium-3 (5×10^{-6}M)	37	86	35	32	70	73

Inhibition of activity in 15 min assays without pre-incubation of enzyme and inhibitor. Percent inhibition was calculated by comparison with parallel assays without inhibitor.

cholinesterase. 2×10^{-6} M BW284C51 (a "true" AChE inhibitor) inhibited the worm cholinesterases more strongly than the horse serum cholinesterase but less than the eel enzymes. These inhibitor results show that the worm enzymes although classifiable by internal comparison, do not fit the dichotomous "true" vs "pseudo" cholinesterase classification scheme that characterizes the vertebrates.

Thermal inactivation

At 45°C, form I was rapidly inactivated (Figure 9A) whereas form II was more stable. At 45°C and at 56°C, forms III and IV were inactivated in parallel (Figures 9B and 9C). The differences between forms I and II, in spite of the similarity of their kinetic properties, demonstrates that size and stability differences need not lead to a measurable effect on the active site.

Detergent sensitivity

Incubation with 0.5% DOC caused an inactivation of forms I and II whereas forms III and IV were more stable (Table 5). This effect occurs in homogenates and could be used for separately assaying forms III and IV activity without requiring fractionation. Tween 80 suppresses, but does not inactivate forms III and IV.

Fig. 9. Thermal Inactivation.

A small aliquot (less than 20 µl) of each separated form was diluted to 1.5 ml of 0.025 M (K)$PO_4^=$, pH 7.0; 1 mg/ml BSA (preincubated for 10 min in a water bath at the inactivation temperature). One hundred µl aliquots were removed at various times and transferred to assay vials on ice. After all samples had been taken, ChE activity was assayed by addition of 10 µl of [^{3}H] -ACh (5.0×10^{-6} M in assay) for 90 min. Activity is recorded as a percent of a control assay. The control was an equivalent dilution into $PO_4^=$ at room temperature.

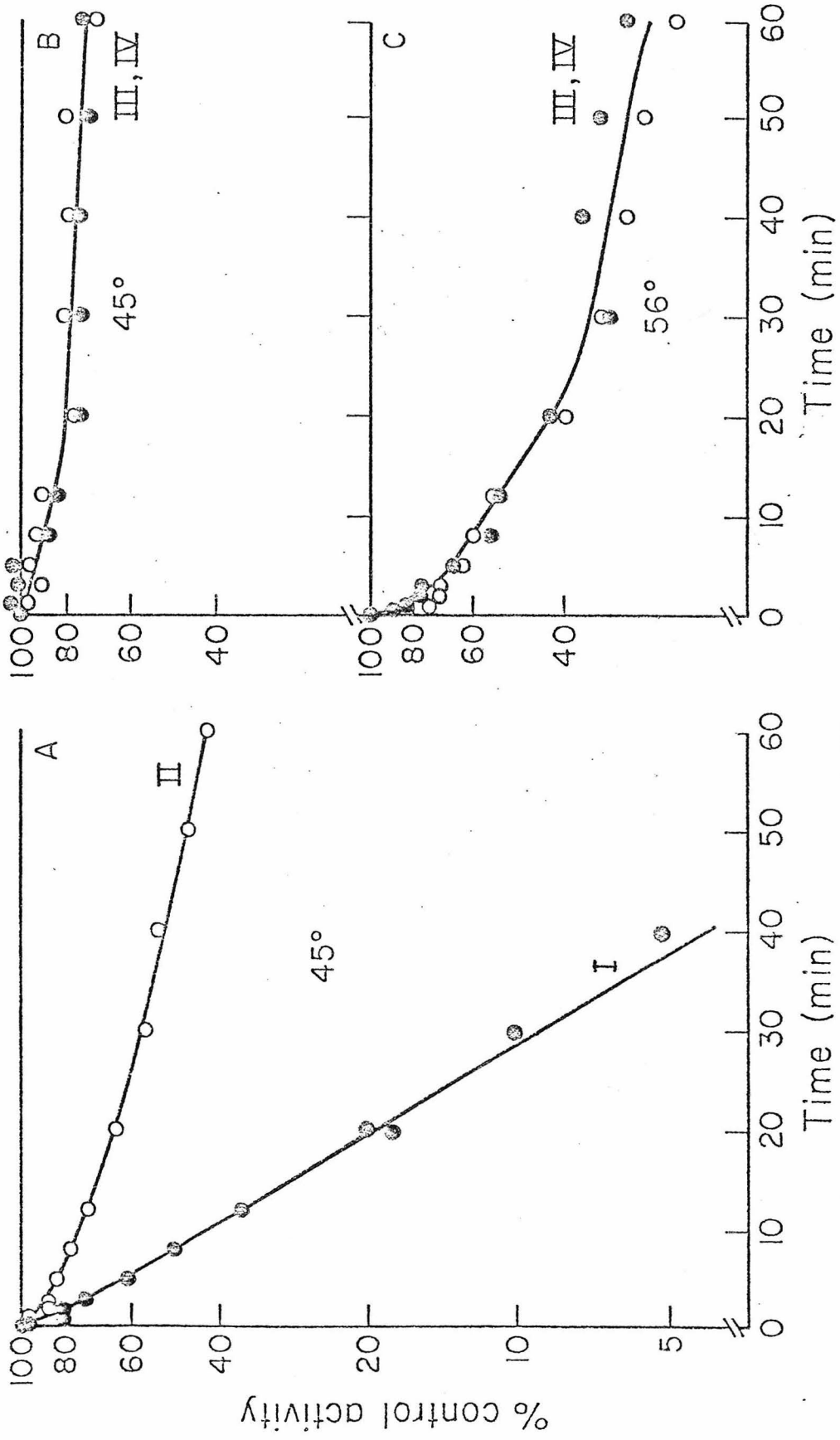
% control activity
100
80
60
40
20
10
5
A
II
I
45°
0
10
20
30
40
50
60
Time (min)
B
III, IV
45°
C
III, IV
56°

Figure 9

Table 5. Detergent Effects on Separated Forms

	Percent Activity Surviving			
Immed Assay	I	II	III	IV
1% Tween 80	90	90	42	42
0.5% DOC	67	84	91	93
Incub. 25 hrs, 4°C				
1% Tween 80	76	90	31	33
0.5% DOC	8	12	51	56

Aliquots of each separated form were diluted into 0.05 M borate, pH8.8, 1 mg/ml BSA divided three ways and maintained at 4°C. Tween 80 was added to 1% in one sample, DOC to 0.5% was added to another. The control received no detergent. Ten µl of each sample was assayed for 90 min with [^{3}H]-ACH at 5.0×10^{-6} M. The assay was repeated after 25 hours incubation at 4°C. Activity is expressed as a percent of the no detergent control.

Discussion

The solubilization of cholinesterase activity from other organisms has received considerable attention without yielding a generally acceptable procedure. Although some activities (e.g. mammalian serum cholinesterase) are seemingly soluble *in vivo*, extraction of homogenates with distilled water or with neutral pH buffers usually releases only small amounts of soluble activity. High ionic strength (Silman & Karlin, 1967; Massoulie & Reiger, 1969; Dudai & Silman, 1974), high pH (Smallman & Wolfe, 1956), divalent ion chelators (Chan, *et al.*, 1972; Wenthold, Mahler & Moore, 1974), nonionic detergents (Ho & Ellman, 1969; Dudai, in press; Lim, Davis & Agranoff, 1971; Hall, 1973; Yamamura, *et al.*, 1973; Jackson & Aprison, 1966), and treatments with proteases (Massoulie, Rieger & Tsuji, 1970; Ho & Ellman, 1969; Dudai, *et al.*, 1972; Betz & Sakmann,1971), collagenases (Hall & Kelly, 1971), and lipases (Lawler, 1964), solubilize portions of the activity in some systems. The extraction of electric eel electric organ AChE with 1 M NaCl is one case where all, or almost all, of the activity is solubilized; in most other cases the extraction is incomplete and additional soluble activity can often be gotten by repetitive extractions or by prolonged incubations at $4^{o}C$, presumably to allow time for "autolytic" digestion. The fractionation of solubilized cholinesterase

activity into multiple molecular forms has often been achieved by velocity sedimentation in sucrose gradients (Massoulie & Rieger, 1969; Hall, 1973; Dudai, in press).

Separable forms may arise from the expression of separate genes (e.g. "pseudo"-cholinesterase vs "true"-acetylcholinesterase) or by the formation of stable associations between a common active-site-containing subunit and other components. These associations may represent sequential stages in the formation of functional complexes, and they may result in the forms having separate localizations and functions. The degradation of these complexes provides another opportunity for production of separable forms of different size.

The solubilization and fractionation of cholinesterase activity by velocity sedimentation in the nematode Caenorhabditis elegans has revealed four molecular forms: form I (5 S), form II (7 S), form III (11 S) and form IV (13 S). Most of form I was released by repeated extraction with 0.05 M borate, pH 8.8. A large portion of the other three forms was retained in the pellet after many washes and was released only after treatment with the anionic detergent deoxycholate (DOC). High ionic strength, EDTA and non-ionic detergents were without effect. All of the forms have been obtained by velocity sedimentation of fresh homogenates after only a few minutes extraction

with DOC suggesting that they are all present *in vivo* and do not represent artifacts of post-homogenization degradation. Once separated, the forms have not been observed to interconvert although specific attempts to interconvert them have not yet been made.

The kinetic properties of the four separated forms (Km, substrate and inhibitor specificity) were compared with one another and with the electric eel ("true") AChE and horse serum ("pseudo") ChE. The four forms of nematode cholinesterase were divided into two classes based upon the kinetic analysis; forms I and II, as compared with forms III and IV, 1) have larger Kms 2) are more specific for acetylthiocholine than for other acylthiocholine substrates and 3) are generally less sensitive to inhibitors. (None of the kinetic differences was very large, and confidence in the differences between them rests largely upon the substantial agreement between the measurements of the two forms within each class.)

All of the nematode enzymes differ significantly from both the eel and the horse serum enzymes. Although the worm enzymes do not show very strong substrate specificity (a characteristic of "pseudo"-cholinesterases) they were all resistant to the specific-ChE inhibitor iso-OMPA at a concentration where even the eel enzyme was substantially inhibited. This failure to adhere to the vertebrate

classification scheme has generally been the case for invertebrate cholinesterases (Chadwick, 1963). Whether the nematode enzymes should be given the designation EC 3.1.1.7 (AChE) or EC 3.1.1.8 (ChE) is unclear. In any case, the kinetic differences between the two classes of forms suggests that their active-site-containing subunit may be different. The selective suppression of forms III and IV with Tween 80 and the selective inactivation of forms I and II with DOC support this suggestion.

Forms III and IV were much more thermostable than were the smaller forms. Forms I and II were significantly inactivated at 45°C whereas 56°C was required to significantly inactivate forms III and IV. It is interesting that although forms I and II have similar kinetic properties, form II was considerably less heat sensitive. Assuming that I and II have a common active-site-containing subunit, this shows that some properties of a complex can be altered without measurably affecting the active site.

The kinetic differences between classes of forms are not large enough to allow selective determinations of the activity of a single class without fractionation. However, selective inactivation of forms I and II with heat or with DOC could be used to allow a selective determination of the more stable forms III and IV.

If forms I and II have a common active-site subunit, it is natural to ask whether form II could be a dimer of

form I. Assuming that both forms are globular (gel filtration suggests that none of the forms are elongated) their molecular weights calculated from sedimentation coefficient would be about 82,000 daltons (form I) and 130,000 daltons (ratio 1.6). This value is considerably less than 2 and suggests that a different component is added to form I to yield form II. Alternatively, the cleavage and release of a portion of form I followed by dimer formation could produce a form II of the appropriate size. The relationship between forms III and IV (estimated molecular weights 275,000 daltons and 343,000 daltons) is proportionally smaller and may involve secondary modifications (e.g. perhaps a polysaccharide component). It would be most interesting to know the size of the active-site-containing polypeptides in the different forms and an approach to this is being made by SDS-polyacrylamide gel electrophoresis after covalent labelling of the active-site serine with [^{3}H]-DFP. In addition, experiments to determine the order and relative rates of reappearance of active enzyme <u>in vivo</u>, after irreversible inhibition with DFP may demonstrate or rule out the possibility that the different forms represent sequential stages in the formation of functional complexes.

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IV Biochemistry and Behavior of a Mutant Nematode Missing Forms of Cholinesterase

Abstract

A screening procedure based upon selective inactivation by sodium deoxycholate (DOC) has been used to screen eighty nine mutants of Caenorhabditis elegans for their relative contents of different forms of cholinesterase. One uncoordinated mutant (BC46) was found which lacked DOC stable activity and this indication that the mutant was missing cholinesterase forms III and IV was confirmed by velocity sedimentation. Biochemical characterization (Kms, substrate and inhibitor specificity and thermal inactivation) of forms I and II from BC46 suggests that they are unaltered. The mutant's rate of growth and fecundity are normal; the most apparent defect was the inability to coordinate the propagation of waves of contraction in the body region of the animal. The motions of the head are less affected. Sensory responses to mechanical, chemical and osmotic stimuli have been demonstrated. After several backcrosses, the behavioral and biochemical defects have not separated and both map to the X-chromosome.

Introduction

When multiple forms of cholinesterase are found in an organism, two related questions naturally arise: 1) do the forms share a common active subunit, and 2) do they have separate functions? A case for which clear answers exist is the case of human "true" and "pseudo" cholinesterases. Here the large differences between the two enzymes in such qualitative properties as Km, substrate specificity, substrate inhibition and inhibitor sensitivity suggest different active subunits and this suggestion is strongly borne out by the existence of human variants with altered pseudocholinesterase (review, La Du & Dewald, 1973). Originally detected by their extreme response to preoperative doses of the muscle relaxant succinylcholine, some of these variants have markedly reduced serum levels of pseudocholinesterase (Kattamis, Davies & Lehmann, 1967; Rubenstein, et al., 1970); in other cases pseudocholinesterase activity in serum (Kalow & Staron, 1957) and in other tissues (Liddell, Newman and Brown, 1962) has altered substrate and inhibitor specificities. Nonetheless, their true acetylcholinesterase is unaltered and, in the absence of succinylcholine, they are asymptomatic, indicating both that the true and pseudocholinesterases have different subunits and that the pseudocholinesterase, in contrast to the true cholinesterase, is quite inconsequential.

In other cases, the evidence is less complete. In

rat diaphragm, for example, where three forms of cholinesterase are found (4 S, 10 S and 16 S), the largest form is selectively localized to the endplate region of the muscle fibers, suggesting that it is the synaptically relevant form (Hall, 1973). However, it is unclear whether this form has the same active subunit as the others, since there is no genetic variant affecting these enzymes; all that can be said at the moment is that it resembles the other forms in its Km (2.0×10^{-4} M, Hall, 1973). The absence of firm subunit evidence is frustrating in this case, since a clear demonstration of a common active subunit would make the important implication that incorporation of this subunit into the synaptically relevant form entails a specific structural change. A genetic variant could also facilitate attempts to determine whether the synaptically relevant enzyme is synthesized in the motoneuron or in the muscle.

In C. elegans, as shown in the previous part, the four separable forms of cholinesterase can be divided into two classes on the basis of differences in qualitative properties (Km, substrate specificity, inhibitor specificity, thermal sensitivity). However, the differences between the classes are not as striking as for the vertebrate true and pseudo cholinesterases, and they might conceivably result from changes arising when a common

active subunit associated with other components. In addition there has heretofore been no method for determining whether these forms have separate functions.

Since a genetic variant would help greatly to resolve these questions in C. elegans, this section describes a screening method for such variants, together with a description of the first variant it has detected, an uncoordinated mutant lacking cholinesterase forms III and IV.

Materials & Methods

Methods for the isolation and study of recessive mutants of Caenorhabditis elegans have been described (Brenner, 1974). The behavioral and morphological mutants analyzed here were isolated as part of a large mutant hunt (Coleman, et al., 1972) using a selection procedure involving failure to execute rapid chemotaxis to bacteria. Mutants used for mapping are from Dr. S. Brenner's laboratory through Dr. R.S. Edgar and Dr. D. Hirsh. Other materials were as described in part III.

Homogenization, extraction, velocity sedimentation, assays for cholinesterase and kinetic characterization experiments were performed as described in part III, Methods. For screening of cholinesterase activity levels in mutants, 1-10 plates of animals were eluted from their growth plates, washed with distilled water and homogenized

by freeze-powdering in liquid nitrogen (0.5-1.0 ml final volume). One hundred µl aliquots of the homogenate were diluted with an equal volume of 0.1 M borate, pH 8.8 and separate aliquots received 20 µl of 10% Tween 80 or 10 µl of 10% DOC. After incubation at 4^{o}C for 12-36 hours, the extracts were assayed for cholinesterase activity.

Parameters of growth and reproduction of mutants were determined with the "3-egg" plate technique described by Byerly, Scherer & Russell (1976). In this procedure, three juvenile nematodes, from synchronously laid eggs, are transferred to a fresh plate and the number and size distribution of the F_1 and a portion of the F_2 progeny are determined using an electronic nematode counter (Byerly, Cassada & Russell, 1975) just as the F_2 generation begins to hatch (~1 week).

Chemotaxis to NaCl gradients was in 9 cm petri plates containing 4 ml of tracking media, the center of which received 1 µl of 5 M NaCl 4-5 hours before the addition of a single nematode (Dusenbery, Sheridan & Russell, 1975; Ward, 1973). The repellent effect of high osmotic strength was tested by placing several animals inside an annulus formed from 30 µl of 5 M NaCl (unpublished procedure of Dr. J. Culotti). Tracks were photographed by placing the petri plate upside down on photographic paper and exposing it to the light from an enlarger.

The mapping of an uncoordinated (unc) mutant (BC46-see Results) located on the X-chromosome was by segregation of the double mutant cis-heterozygote with the markers lon-2 and dpy-7. In general the production of double mutants for X-linked genes is prevented by the inability of the hemizygous mutant males to mate with hermaphrodites. However, males of the X-linked lon-2 allele E678 mate well, as do the (non-dumpy) males of the temperature sensitive X-linked dpy-7 allele E1324 produced by growth at the permissive temperature (20°C). The double mutant was obtained by mating homozygous unc hermaphrodites with hemizygous lon or dpy males. From this cross, clones of unc +/+lon (or +dpy) hermaphrodites were established and their long (or dumpy) progeny were further cloned. At twice the recombination frequency these clones were heterozygous at the uncoordinated locus and segregated the homozygous double mutant which was cloned to establish a pure double mutant culture. The double mutant was then mated with wild type males to produce the double mutant cis-heterozygote.

Results

Screening procedure

In designing a screening procedure to detect genetic variants of C. elegans cholinesterase, an important consideration was to have a method which would monitor the two

major cholinesterase classes (forms I and II versus forms III and IV) selectively. Among the class differences reported in part III that might be exploited for this purpose, differences in thermal sensitivity and detergent sensitivity seemed well suited, since forms III and IV could be separately monitored (after inactivation of forms I and II) without prior fractionation. Detergent sensitivity was chosen and in order to see whether the class differences detected in the separated forms would hold in the whole, mixed homogenate, a homogenate was extracted with 0.5% sodium deoxycholate (DOC) and assayed at intervals thereafter. As shown in Figure 1A, there was a rapid initial loss of activity until about 12 hours, when a stable level was reached at about 30-40% of the initial activity. Velocity sedimentation of homogenate extracted for 30 min or for 8 hours in 0.5% DOC (Figure 1B) indicated that the rapid loss had been, as expected, primarily of the slower sedimenting forms I and II.

A standard screening procedure based on these observations was then devised. Using paired aliquots of homogenate incubated in 1% Tween (suppresses forms III and IV) or 0.5% DOC (inactivates forms I and II), a total of 89 mutants were screened for possible defects. These included 36 mutants resistant to cholinesterase inhibitors, and 53 behavioral or morphological mutants isolated in a large previous mutant hunt (Coleman, et al., 1972). Figure 2

Fig. 1. Inactivation of cholinesterase activity in homogenate by incubation with 0.5% DOC.

A. Nematode homogenate was diluted to 0.05 M borate pH 8.8; 0.5% DOC and stirred at 4^{o}C. At specific times, an aliquot was assayed with 5.0×10^{-5} M ACh. Cholinesterase activity was plotted as a percent of that measured 5 min after dilution.

B. Homogenate was diluted as in A and after 30 min (top gradient) or after 8 hours (lower gradient) at 4^{o}C, 100 µl aliquots were loaded unto 4.5 ml 5-20% sucrose gradients (0.05 M borate; pH 8.8; 0.1% Tween 80) with 0.5 ml Renografin cushions. After centrifugation for 4 hours at 65,000 rpm, fractions were collected and 10 µl of each was assayed for 1 hour with 5×10^{-5} M ACh. The blank was 0.4×10^{3} cpm; complete conversion 21.7×10^{3} cpm.

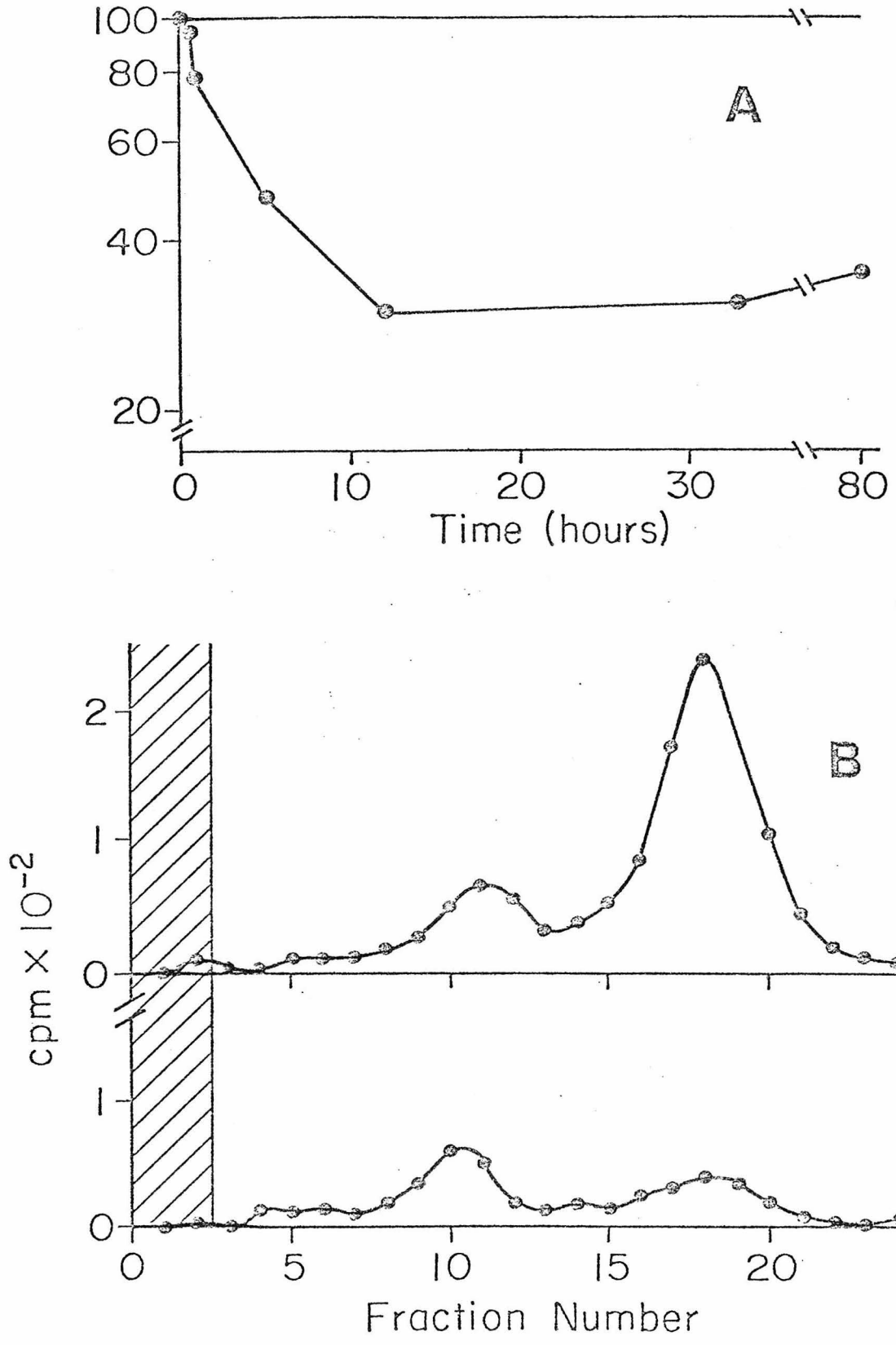

100
80
60
40
20
A
0
10
20
30
80
Time (hours)
2
1
0
1
0
B
cpm X 10⁻²
0
5
10
15
20
25
Fraction Number

Figure 1

Fig. 2. DOC inactivation of mutant extracts.

Ten plates of animals for each strain to be assayed were eluted, washed with distilled water and freeze powdered in 1 ml. One hundred µl aliquots of homogenate were diluted with an equal volume of 0.1 M borate, pH 8.8. Separate aliquots received 20 µl of 10% Tween 80 or 10 µl of 10% DOC. Ten µl aliquots of the extracts were assayed for 5 min with 5.0×10^{-5} M ACh at about 12 hour intervals. Activity (u/ml) was plotted versus time of assay. The DOC extract of one mutant (BC46) was devoid of activity after about 24 hours.

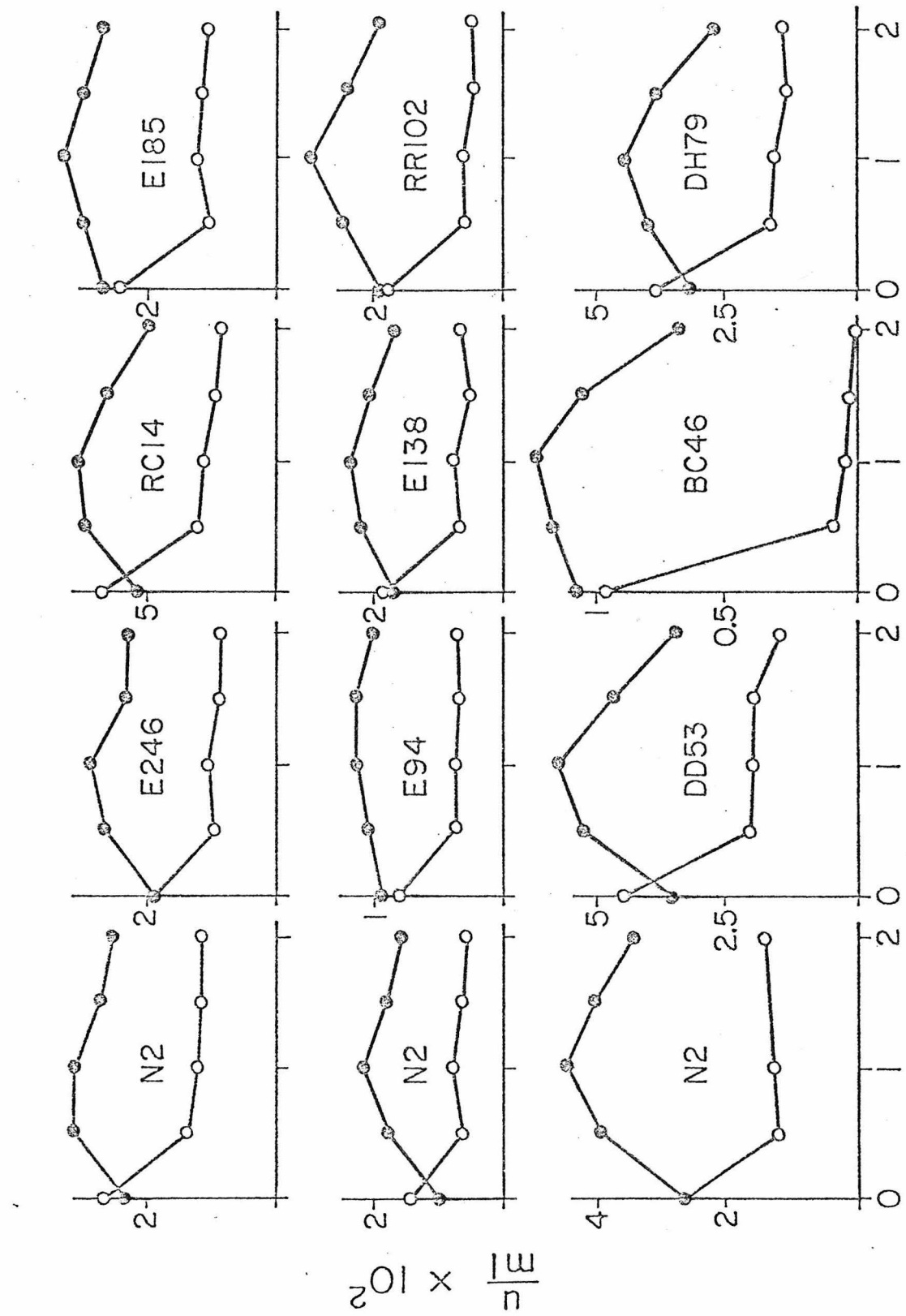

N2
E246
RC14
E185
N2
E94
E138
RR102
N2
DD53
BC46
DH79
Time (days)
$\frac{m}{m1} \times 10^2$

Figure 2

Fig. 3. Stable DOC/Tween ratio in mutant extracts.

Homogenates were extracted with 0.05 M borate, pH 8.8 and Tween (1%) or DOC (0.5%) and assayed after 24-27 hours. Activity measured in the DOC extract is expressed as a fraction of the activity measured in the Tween extract. The arrows represent N2 controls.

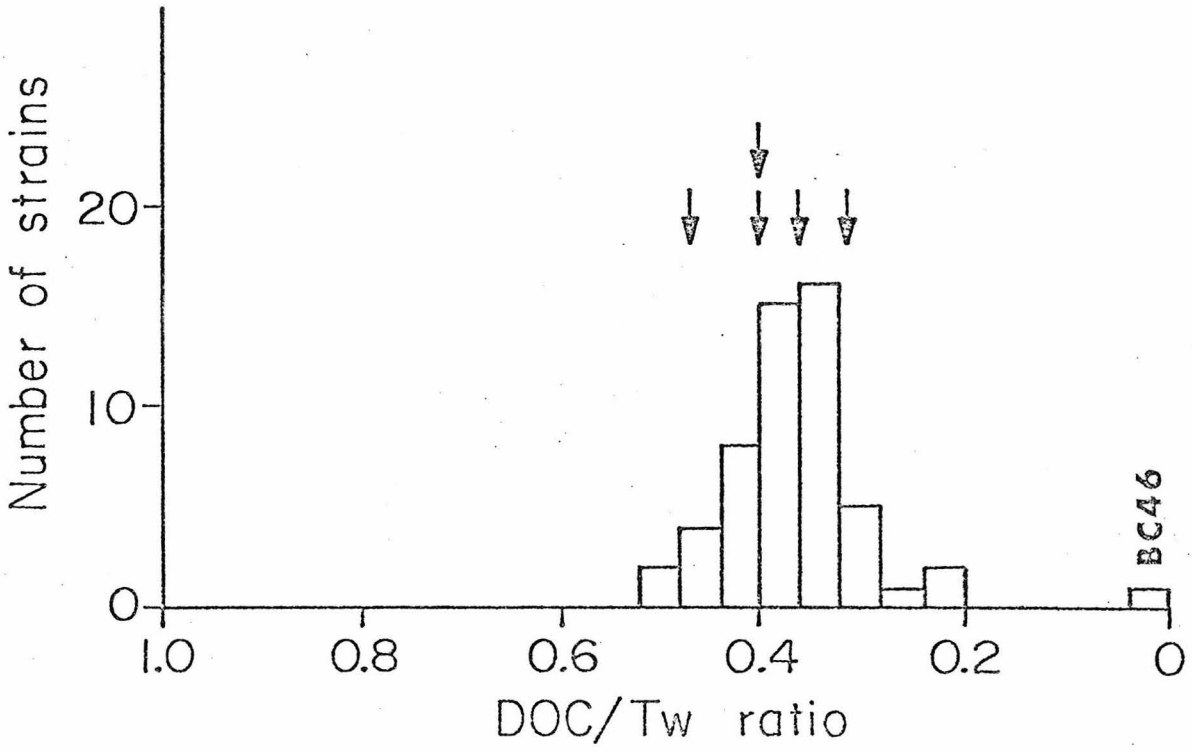


Figure 3

shows the screening results for one series of mutants, including the one mutant (BC46) which was found to be significantly altered. For wild type and for most of the mutants the activity measured in Tween rose slightly and then gradually fell, whereas activity in the DOC extract showed the rapid loss and plateau behavior of Figure 1A. For BC46, however, there was virtually no DOC-resistant activity, and as the histogram of Figure 3 shows, BC46 was unique in this respect.

BC46 cholinesterase

The implication of the initial screening result was that BC46 might lack cholinesterase forms III and IV or that these forms if present were as sensitive to DOC as were forms I and II. Direct velocity sedimentation of a BC46 homogenate showed no sign of forms III or IV (Figure 4) indicating that if forms III and IV were present, they were either abnormally sensitive to homogenization or abnormally tightly bound to pelletable debris. Short-term extraction with DOC followed by rapid sedimentation into gradients containing Tween 80, a procedure designed to maximize the amount of solubilized cholinesterase (as explained in part III) also failed to reveal any BC46 activity sedimenting as forms III or IV (Figure 5).

Larger amounts of BC46 were grown in liquid cultures and fractionated by repetitive extraction in 0.05 M borate, pH 8.8 (Figure 6A) releasing about 50% of the total

Fig. 4. Velocity sedimentation of N2 and BC46; extracted with 0.05 M borate, pH 8.8.

Fresh homogenates of (top gradient) N2 and (lower gradient) BC46, were diluted three fold with 0.1 M borate, pH 8.8. After stirring 24 hours at 4^{o} C, 1 ml aliquots of a 1500 g, 5 min supernatant was loaded unto 38 ml 5-20% sucrose gradients (0.05 M borate). The gradients were centrifuged at 27,000 rpm for 29 hours. Fractions were collected and 10 µl of each was assayed for 45 min with 5×10^{-5} M ACh. The blank was 0.4×10^{3} cpm; complete conversion 16.7×10^{3} cpm.

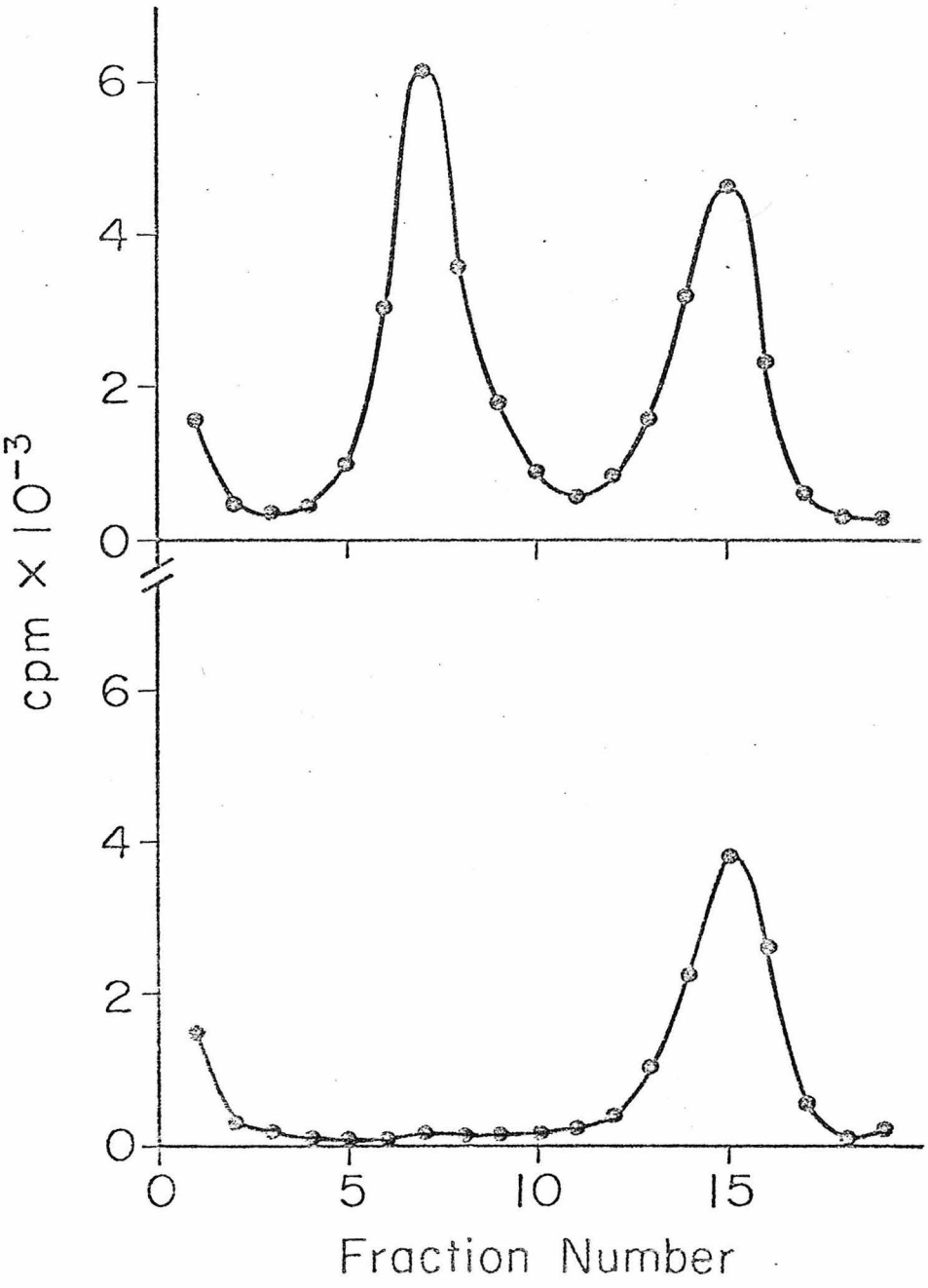


Figure 4

Fig. 5. Velocity sedimentation of N2 and BC46; extracted with 0.05 M borate, pH 8.8, 0.2% DOC.

One ml of (top gradient) N2 and (lower gradient) BC46 homogenate was diluted with 0.8 ml of 0.1 M borate, pH 8.8. At 4°C, DOC was added to 0.2% and, after 2 min, a 1500 g, 1 min supernatant was prepared and 100 µl aliquots were loaded unto 5 ml 5-20% sucrose gradients in 0.05 M borate, pH 8.8, 0.1% Tween 80. Gradients were centrifuged for 4.5 hours at 65,000 rpm; fractions were collected and 10 µl of each was assayed for 10 min with 5×10^{-5} M ACh. The blank was 1.1×10^{3} cpm; complete conversion 32.0×10^{3} cpm.

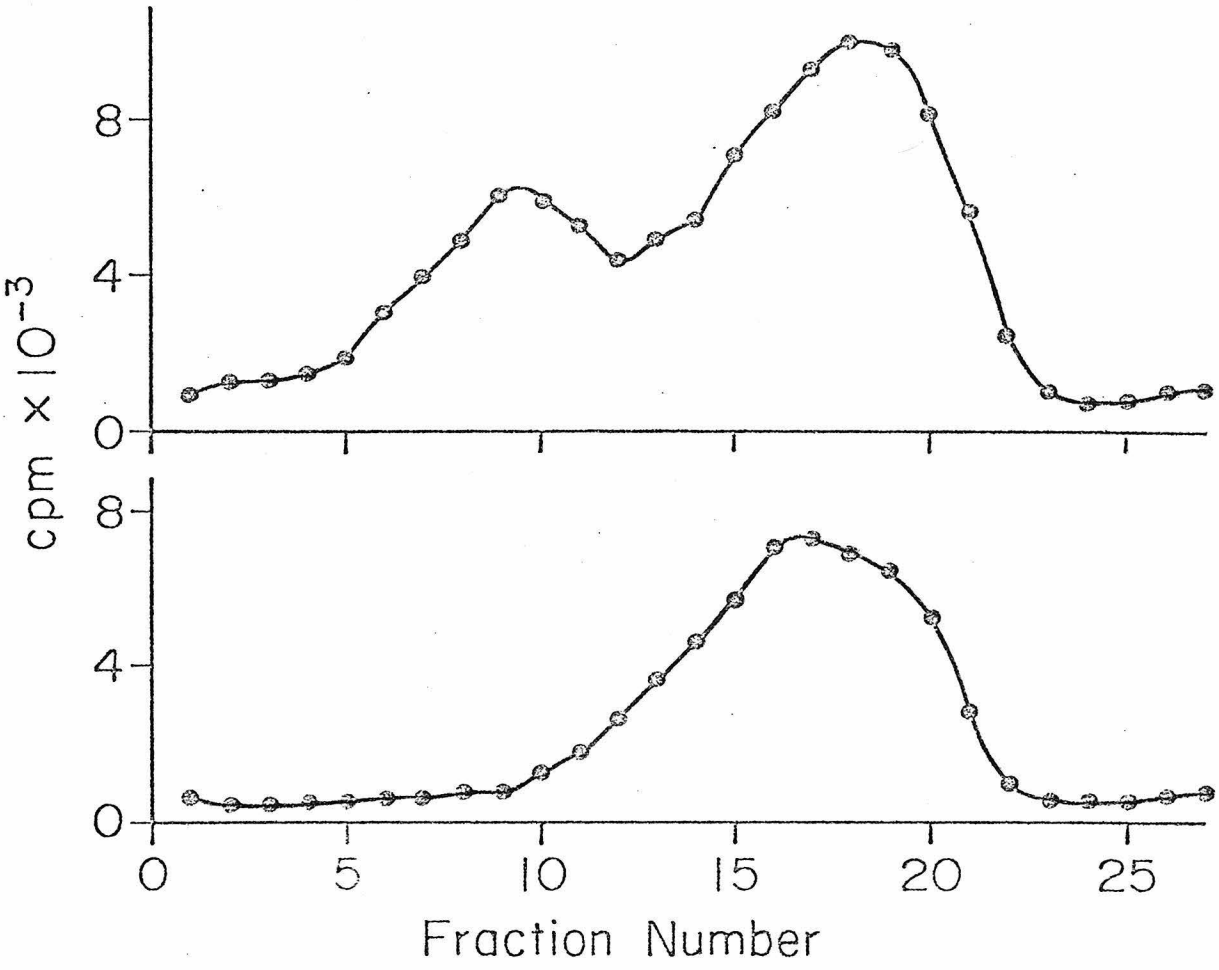


Figure 5

activity to high speed supernatants. When these supernatants were examined by velocity sedimentation (Figure 6B), the activity sedimented primarily as a 5 S peak, but the later washes contained substantial amounts of activity at 7 S as well. Treatment of the pellet with 0.2% DOC released additional activity most of which sedimented at 7 S (bottom gradient - Figure 6B). The 5 S (BC46 - form I) and the 7 S (BC46 - form II) activities were separately pooled and characterized with experiments parallel to those performed on the separated forms of the wild type enzyme (part III - Figures 8 and 9, Tables 3 and 4). Forms I and III from wild type, were included in the experiments for comparison.

Km

Forms I and II from BC46 have Km's of 9.1×10^{-5} M and 8.3×10^{-5} M respectively, both quite close to the Km of wild type form I (Figure 7). As expected, the wild type form III has a significantly lower Km. In this determination, several higher substrate concentrations were used than were used for the Km determinations in part III and substrate inhibition was demonstrated for all forms.

Substrate specificity

The relative specificities of BC46 forms I and II for acylthiocholine substrates are quite comparable with those of wild type form I and different from those of

Fig. 6. Repeated extraction of BC46.

About 8 gm of fresh nematodes (BC46) were homogenized and thawed into 5 volumes of distilled water and a 40,000 rpm, 30 min supernatant was prepared (S40K-1). The pellet was rehomogenized (by freeze-powder) in 10 ml 0.05 M borate, pH 8.8 and another supernatant was prepared (S40K-2). This pellet was resuspended, rehomogenized and recentrifuged five more times over several days (yielding S40K-3 thru S40K-7). In panel A, the total units of cholinesterase activity extracted was plotted against the time of supernatant preparation. The dashed lines above S40K-5 and S40K-7 represent assays of activity remaining in the pellet. Panel B shows sucrose gradients of aliquots of all the S40Ks and (bottom gradient) the final pellet after extraction with 0.2% DOC for 2 min). The activity measured in each fraction was converted to units/ml and multiplied by the total volume of the S40K before plotting against fractional position (corrected to a sedimentation run of 8 hours at 65,000 rpm).

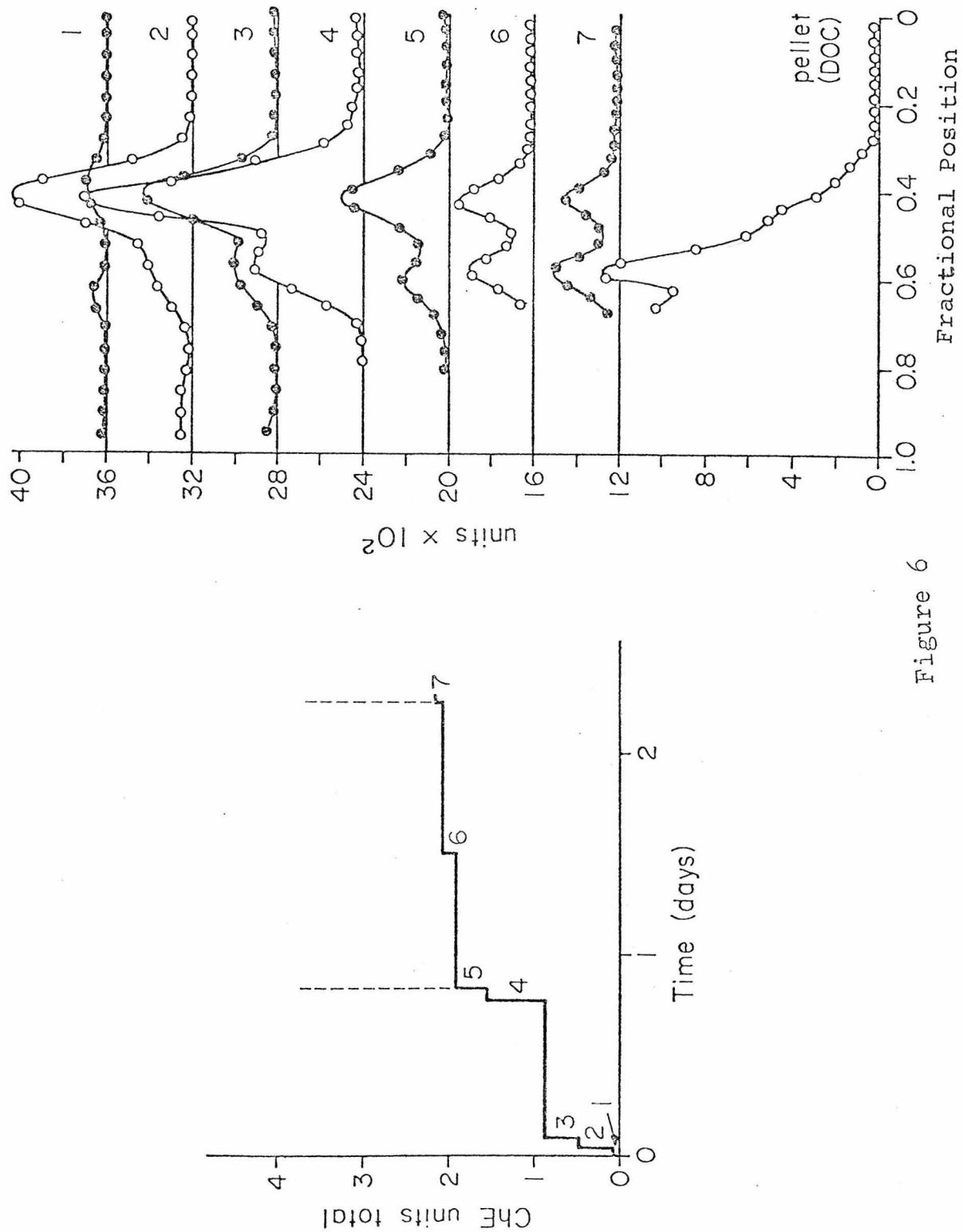

units x 10²
40
36
32
28
24
20
16
12
8
4
0
1.0
0.8
0.6
0.4
0.2
0
Fractional Position
1
2
3
4
5
6
7
pellet
(DOC)
CHE units total
4
3
2
1
0
Time (days)
0
1
2
1
2
3
4
5
6
7

Figure 6

Fig. 7. Km for acetylcholine.

Forms I and II, separated from extracts of BC46 and the wild type cholinesterase forms I and III were assayed with 10 concentrations of ACh ranging from 2×10^{-6} M to 1.5×10^{-3} M. The Km was calculated as a linear regression of the measurement with ACh less than 4×10^{-4} M. The standard deviations of the Km determinations were BC46-form I, $\pm 0.5 \times 10^{-5}$ M; BC46-form II, $\pm 0.6 \times 10^{-5}$ M; N2-form I, $\pm 0.5 \times 10^{-5}$ M; N2-form III, $\pm 0.1 \times 10^{-5}$ M.

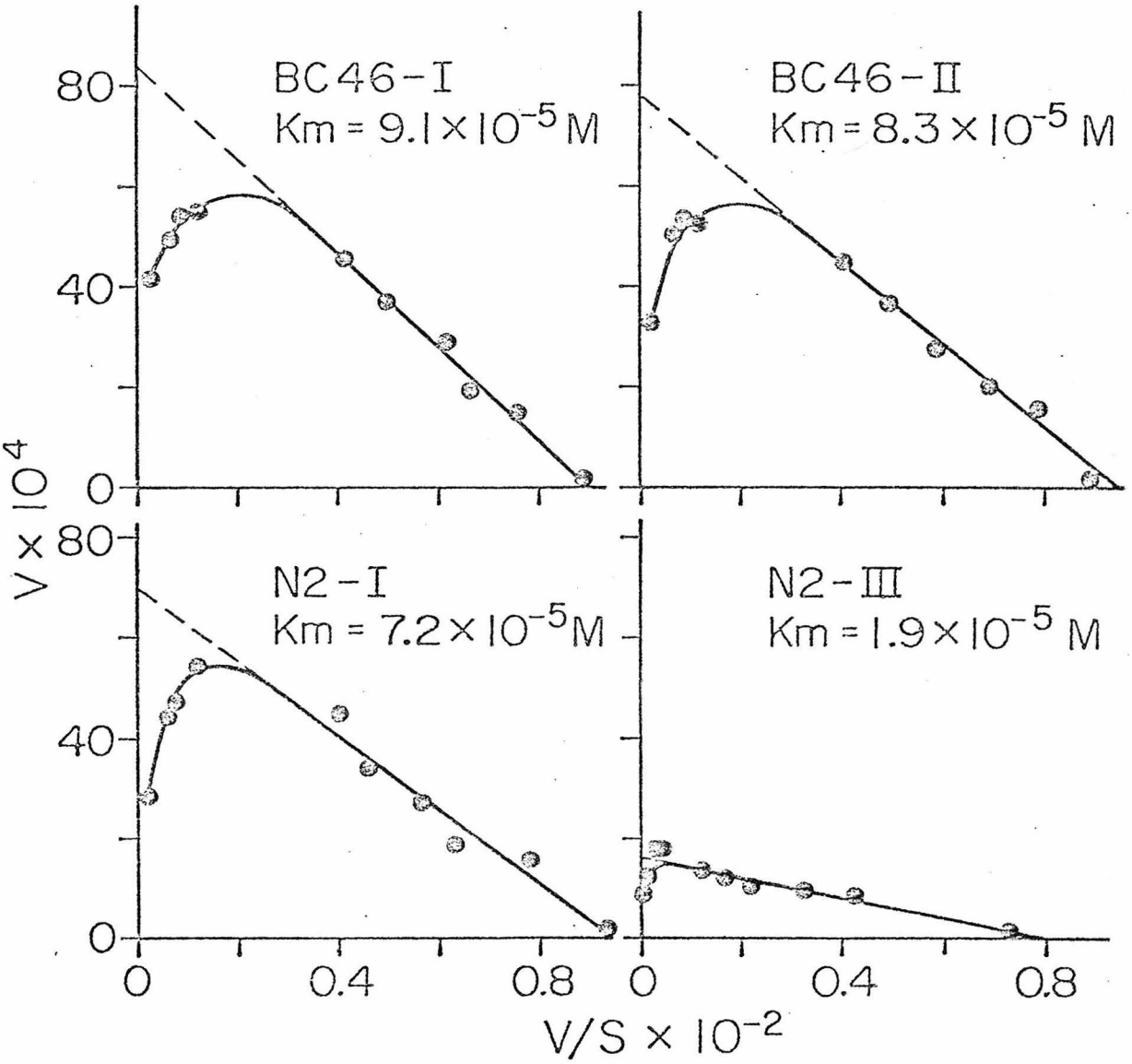

BC46-I
Km = 9.1×10⁻⁵ M
BC46-II
Km = 8.3×10⁻⁵ M
N2-I
Km = 7.2×10⁻⁵ M
N2-III
Km = 1.9×10⁻⁵ M
V × 10⁴
80
40
0
0
0.4
0.8
V/S × 10⁻²

Figure 7

wild type form III as shown in Table 1.

Inhibitor specificity

The same pattern was seen when the mutant enzymes were examined for inhibitor specificity (Table 2). BC46 forms I and II and the wild type form I were inhibited to about the same extent by the concentrations of inhibitors chosen, whereas the wild type form III was generally more strongly inhibited.

Thermal inactivation

At 45.1°C, forms I from BC46 and from wild type were inactivated in parallel (Figure 8). BC46 form II was more stable, its inactivation paralleling the wild type form II inactivation (part III, Figure 10A).

The biochemical characteristics of the separated forms I and II from BC46 are nearly identical to the same form in the wild type and significantly different from wild types forms III and IV. (Whether the small differences observed between the two forms I are a reliable indication of additional heterogeneity remains to be seen).

BC46 development

To obtain quantitative measures of the size, the rate of development and the fecundity of BC46, the progeny descendant from three synchronously laid hermaphrodites were counted and sized with an electronic nematode counter about one week (two generations) after the three animals were placed on a fresh growth plate. BC46 was,

Table 1. Acyl-thiocholine Substrate Specificity of BC46 Forms

Substrate	% Activity of ChE form			
	BC46		N2	
	I	II	I	III
acetylthiocholine	100	100	100	100
acetyl-β-methylthiocholine	39	44	44	63
propionylthiocholine	45	48	60	99
propionyl-β-methylthiocholine	26	28	35	99
butyrylthiocholine	19	23	24	64

All substrates were at 1 mM in the assay. Activity (ΔOD_{412}/min) is recorded as percent of the activity measured with acetylthiocholine.

Table 2. Inhibitor Specificity of BC46 Forms

Inhibitor	% Inhibition of BC46 I	BC46 II	N2 I	N2 III
neostigmine (2×10^{-7} M)	16	18	31	83
temik (2×10^{-5} M)	23	23	33	71
DFP (10^{-6} M)	21	23	30	78
paraoxon (10^{-6} M)	14	16	18	24
tensilon (2×10^{-6} M)	26	29	36	69
BW284C51 (2×10^{-6} M)	51	68	67	99
TEA (5×10^{-4} M)	65	65	61	62
hemicolinium-3 (5×10^{-6} M)	23	26	23	77

Inhibition of activity in 15 min assay with 5×10^{-6} M ACh (without preincubation of enzyme and inhibitor). Percent inhibition was calculated by comparison with parallel assays without inhibitor.

Fig. 8. Thermal inactivation.

Aliquots (10 or 20 µl) were diluted into 1.5 ml 0.025 M $(K)PO_4^{=}$, pH 7.0; 1 mg/ml BSA which had been prewarmed to 45.1°C. One hundred µl aliquots were removed at specific times thereafter and transferred to assay vials on ice. After all time points were taken, activity was assayed and recorded as a percent of control (an equivalent aliquot diluted into buffer at room temperature).

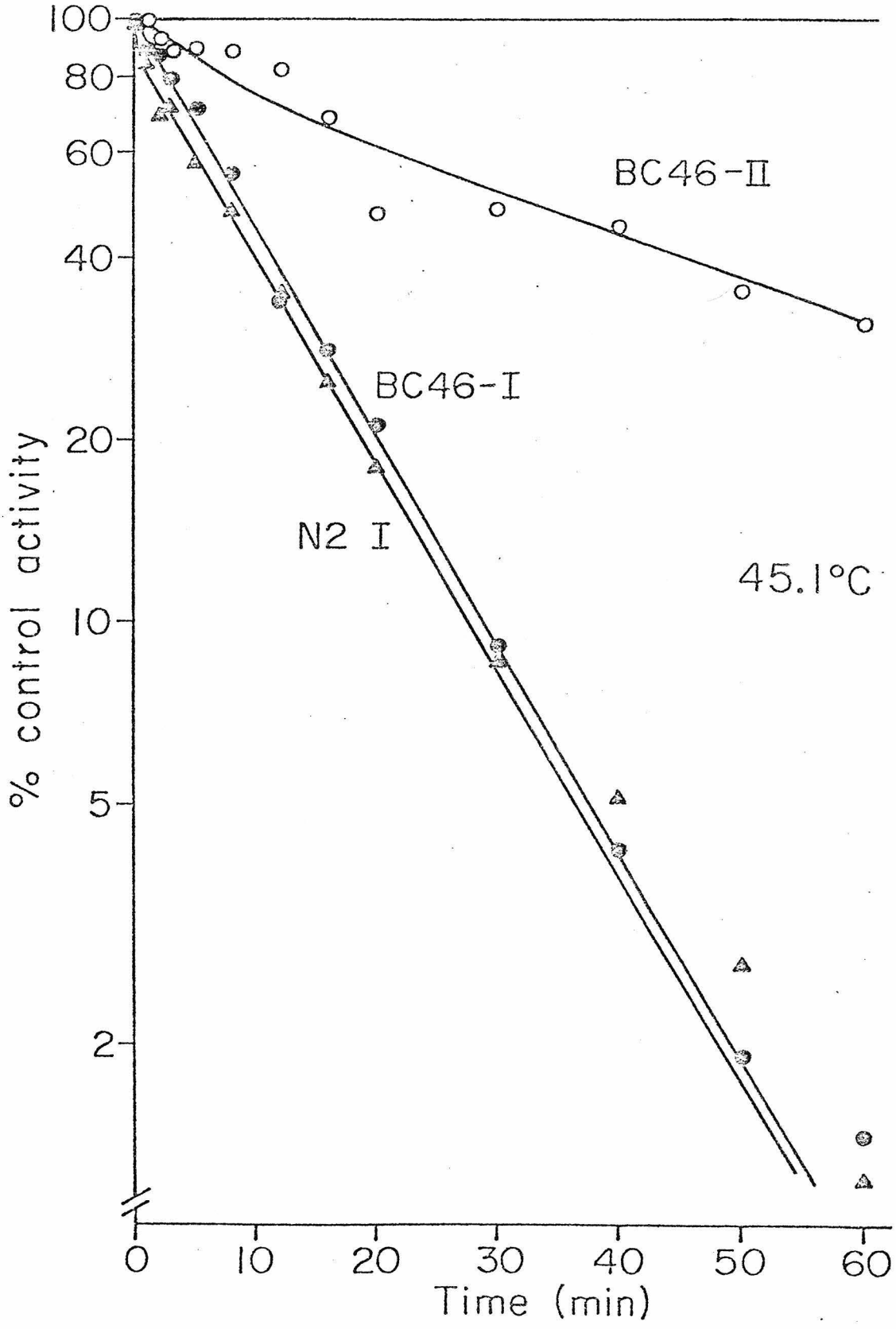

100
80
60
40
20
10
5
2
% control activity
BC46-II
BC46-I
N2 I
45.1°C
0
10
20
30
40
50
60
Time (min)

Figure 8

on the average, 106% the size of wild type; they grew at 95% the rate and produced 97% of the expected number of progeny.

BC46 behavior

The behavioral defect in BC46 prevents the continuous wave propagation necessary for sustained movement. Although waves of contraction are often seen in the head propagating backwards, these waves rarely pass the vulva and usually die out about one-fifth of the way down the body. The head is much more normal and active than the body. The low amplitude searching movements involved in feeding seem unaffected and the pharnyx pumps normally. Especially when excited by mechanical stimuli, the head undergoes a dorsal-ventral swinging with a frequency very similar to the frequency of wave propagation in the wild type (Table 3).

When tapped on the snout with a toothpick, wild type nematodes respond immediately, moving backward by passing forward travelling waves. During backward movement the low amplitude searching movements of the head are stopped. This head-tap-response (htr) lasts about 5 seconds (Table 3) after which the animal reverses and begins to move forward again. Tapping BC46 on the head produces an overall muscular contraction, a short backward movement and a curling of the tail which suggests the beginning of

a forward travelling wave. This "pose" lasts about 5 seconds during which the head searching movements cease (Table 3) and is followed by relaxation. If during a head-tap-response, the tail is tapped, the wild type interrupts its backward movement and immediately moves rapidly forward. Similarly in BC46, if the tail is tapped during the "pose" response to head-tap, head swinging movements resume immediately.

BC46, when placed in radial gradients of chemical attractants (e.g. NaCl) showed strong chemotactic responses (Figure 9A), although moving slowly. The repulsive response caused by high osmotic strength was seen as well (Figure 9B).

These observations suggest that the nervous system of BC46 is quite intact, being able to sense a variety of stimuli so as to produce essentially normal (if slowly executed) behavioral responses. Furthermore, in the case of the mechanical responses, the duration of the behavioral response to head-tap, and its reversal by a second stimuli (tail-tap), were unaltered even though the mutant's locomotory defect prevented any effective response to the stimuli.

BC46 genetics

The initial isolate of BC46, in addition to being uncoordinated, failed to grow at 25°C. When backcrossed

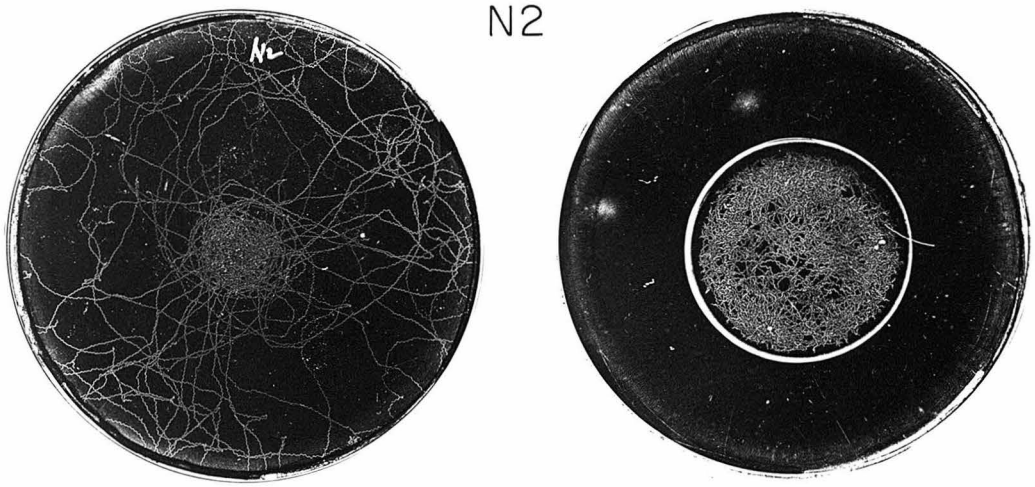
Table 3. Behavior and Mechanical Responses of BC46.

Response	N2	BC46
frequency of waves (sec^{-1})	0.59 ± 0.07 (n=10)	0.52 ± 0.14 (n=10) 0.60 ± 0.12 (n=10)
duration of head-tap-response (htr) (sec)	4.4 ± 1.6 (n=30)	4.7 ± 2.1 (n=27) 5.8 ± 2.0 (n=10)
frequency of stopping htr with tail-tap	0.85 (n=20)	0.90 (n=10)

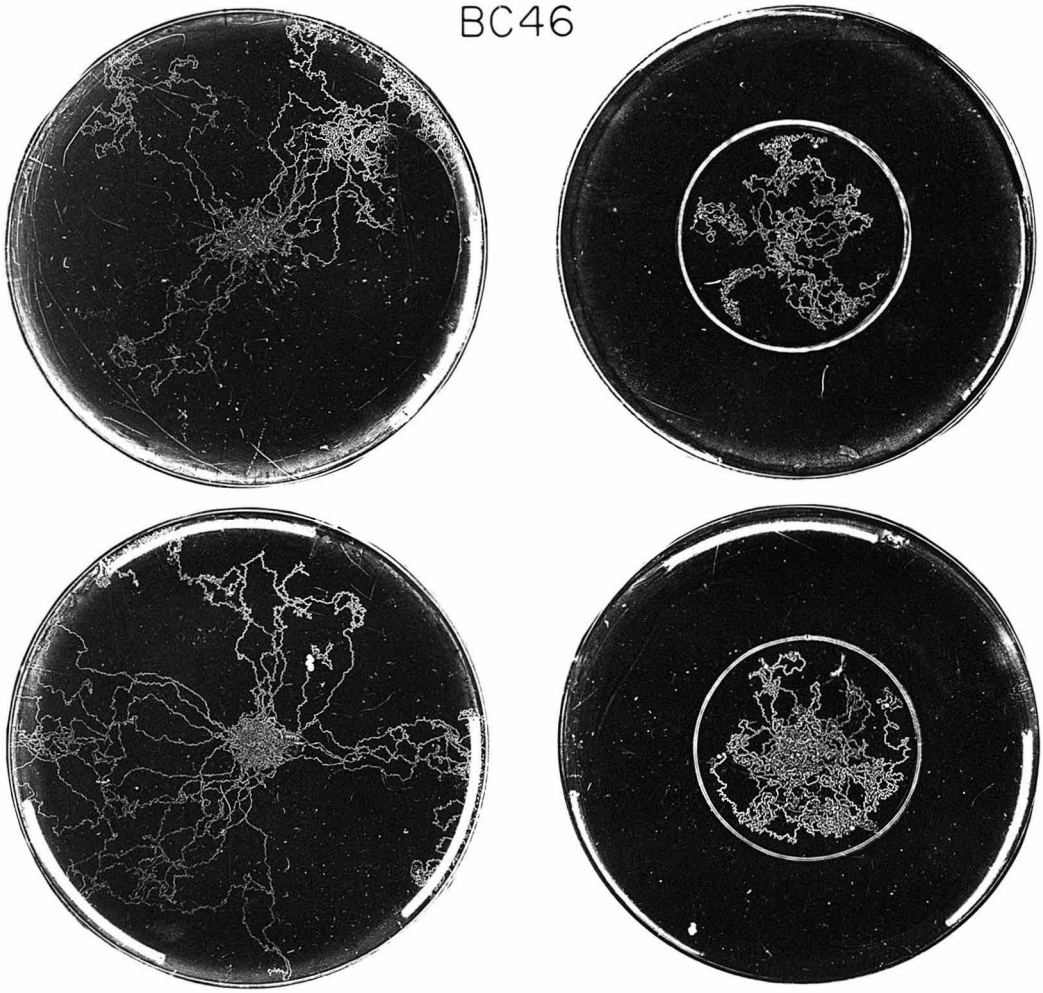
Fig. 9. Chemotaxis and osmotaxis.

Tracks made by N2 or BC46 hermaphrodites were photographed after A. 11 hours on a radial gradient of NaCl (1 µl of 5 M NaCl allowed to diffuse for 4 hours before addition of single nematode) or B. 2 hours inside an annulus of high ionic strength (30 µl of 5 M NaCl, 4 nematodes placed in the center immediately). Osmotaxis studies were developed and performed here by Dr. J. Culotti.

N2



BC46



to N2 males and reisolated (as F_2 uncoordinated hermaphrodites), only three of twelve clones retained the ts growth characteristic. In addition, all of the F_1 males of the backcross were uncoordinated. From this, it would appear that the unc mutation is sex-linked, whereas the ts growth results from a separate, autosomal mutation. Twenty uncoordinated clones were tested for the presence of cholinesterase forms III and IV by the DOC inactivation procedure. None were found with DOC stable activity. The absence of forms III and IV was confirmed in five additional backcrosses by velocity sedimentation.

Linkage to the X-chromosome was confirmed by segregation of the cis heterozygote of BC46 and two X-linked markers - lon2 and dpy 7 (Table 4). BC46 was clearly linked, but not very tightly, to both loci. More precise mapping will require more tightly linked markers, but these preliminary results suggest a map position on the right arm of the X-chromosome (Figure 10). This region of the published genetic map (Brenner, 1974) contains three unc loci. The unc7 allele E5, the unc9 allele E101 and the unc3 allele E151 have all been examined for DOC resistant cholinesterase activity and all three were found to be normal in this regard (Table 5).

Discussion

Mutants altered in the distribution of the separable

Table 4. Segregation of BC46 heterozygotes

BC46/+	+	unc		
	110	40		
BC46-E678/++	++	unc lon	unc +	+ lon
	509	69	136	123
BC46-E1324/++	++	unc dpy	unc +	+ dpy
	612	161	91	96

Fig. 10. Genetic mapping of BC46.

The genetic map of the X chromosome is reproduced from Brenner, (1974). The recombination frequency between BC46 and 1) the E678 allele of lon2 and 2) the E1324 allele of dpy7 suggest that BC46 is located on the right arm of the chromosome.

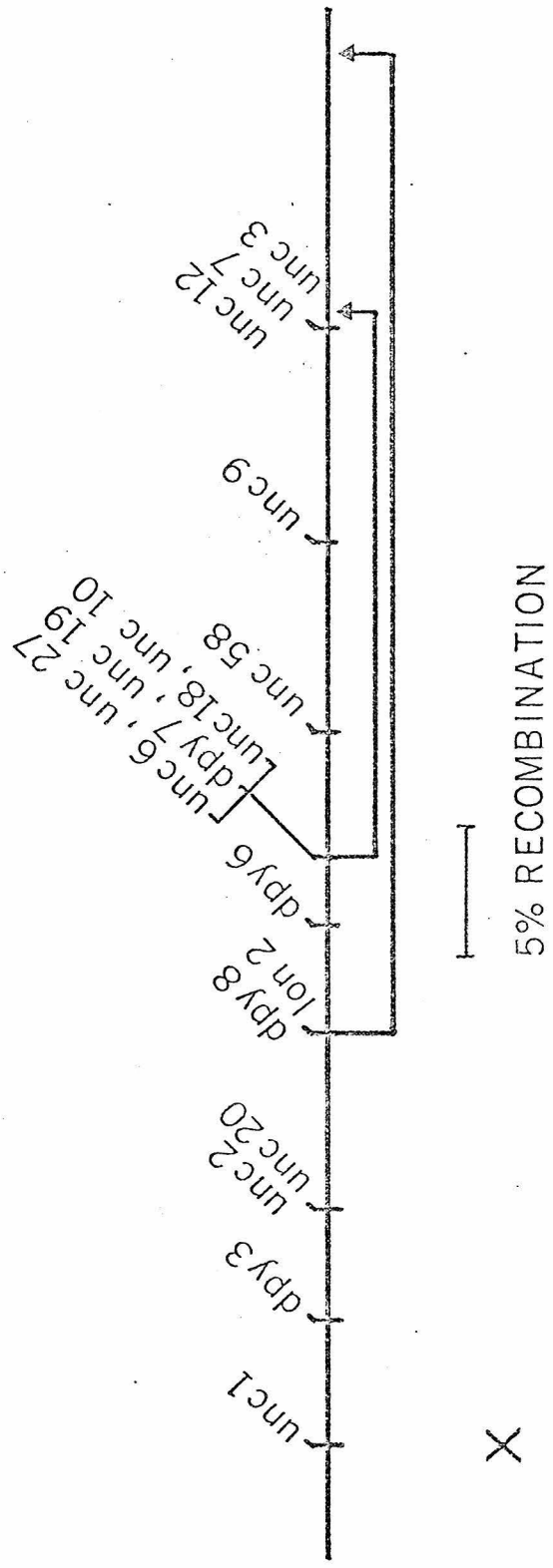

unc1
dpy3
unc20
unc2
dpy8
lon2
dpy6
unc6, unc27
dpy7, unc19
unc18, unc10
unc58
unc9
unc12
unc7
unc3
5% RECOMBINATION
X

Figure 10

Table 5. DOC/Tw Ratio for X-linked Markers.

Locus	Allele	Activity in (u/ml x 10^3) Tween 80	DOC	Ratio DOC/Tw
	N2	6.2	1.3	0.22
	$BC46b_2A$	7.5	0.2	0.03
unc7	E5	19.8	4.8	0.24
unc9	E101	16.6	4.5	0.27
unc3	E151	2.3	0.6	0.26
unc1	E94	14.2	5.1	0.36
	tcfr2	30.5	9.7	0.31

Cholinesterase activity measured after 24 hrs incubation of homogenate in 0.5% DOC or 1% Tween 80.

$BC46b_2A$ is a second backcross of BC46.

forms of cholinesterase activity can provide important constraints on the many possible interrelationships of the different forms. We have screened mutants of Caenorhabditis elegans, for such defects and have found an uncoordinated mutant (BC46), mapping to the right arm of the X-chromosome, which lacks cholinesterase forms III and IV. The identification of this defect was facilitated by the use of a selective inactivation procedure which allowed the separate determination of cholinesterase forms III and IV without requiring fractionation. We have not been able to separate the behavioral phenotype and the biochemical defect by backcrossing to wild type. A direct measure of their recombination frequency ($< 2\%$) suggests that they are either very tightly linked or effects of a single lesion. Revertants have not yet been obtained.

Biochemical characterization (Kms, substrate and inhibitor specificities, thermal inactivation) of the forms retained in BC46 (forms I and II) showed that they were unaltered compared to comparable wild type forms I and II. These results support the suggestion, advanced initially after comparison of the kinetic properties of all four forms isolated from the wild type, that the active-site-containing subunit of forms I and II is a gene product different from that of forms III and IV. BC46 could

represent a lesion in the structural gene for forms III and IV cholinesterase. For the moment, this must remain a suggestion as it is possible that the kinetic differences are secondary effects on a common active-site which occur as a result of converting forms I and II into the larger forms. In this interpretation, BC46 could represent a lesion which prevents the conversion of forms I or II to the forms III and IV.

The behavioral defect of BC46 is apparently limited to the inability to properly coordinate continuous passage of waves of muscular contraction in the body region. Sensory responses to mechanical, chemical and osmotic stimuli have all been demonstrated and the capacity of the nervous system to integrate spatially distinct mechanical stimuli was shown by measuring the duration of the response to head-tap and its suppression by tail-tap. Since the animal does occasionally pass an isolated, well coordinated wave of contraction down its entire length, the muscle does not appear to be defective. This suggests a functional localization of cholinesterase forms III and IV to the body region. If other areas of the nervous system contain cholinergic synapses, it seems likely that they utilize cholinesterase forms I or II.

The apparent normal coordination of muscular contraction by BC46 in the head suggests that the control of

contraction is different here than in the body. One possibility is that the head muscles contain an autorhythmic pacemaker, independent of direct neuronal control.

When C. elegans is treated with externally effective cholinesterase inhibitors (e.g. temik, DFP, paraoxon), the animal becomes extremely hypercontracted. This phenotype was presumed to result from cholinesterase inhibition causing an excessive buildup of acetylcholine, leading to continuous contraction of the somatic musculature. Excessive hypercontraction did not result from the mutational elimination of cholinesterase forms III and IV in BC46. This may be because the remaining cholinesterase is sufficiently widespread to prevent too large of an increase of ACh. Alternatively, the hypercontraction may result from the effects of the inhibitors on other enzymes (e.g. DFP inhibition of ATPase - Hokin & Yoda, 1964). With BC46 in hand, it may be possible to design selective schemes for isolating mutants affecting the remaining cholinesterases, which would allow a precise determination of the behavioral effect of eliminating cholinesterase activity without the unintended side effects from the action of inhibitors on other components.

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