STUDIES ON ACETYLCHOLINESTERASE AND CELL WALL PROTEINS IN *PHASEOLUS VULGARIS* L.

by

DONALD HOLMES MANSFIELD

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Department of BOTANY

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date September 15, 1977

ABSTRACT

Acetylcholinesterase (AchE) activity was identified in roots and hypocotyls of etiolated Phaseolus vulgaris L. by means of a colorimetric assay which included the cholinesterase inhibitor neostigmine as a control. An inhibitor of this activity was observed in tissue homogenates but was removed by dialysis. Greater than 95% of the activity in the hypocotyl was localized in the cell walls. The enzyme was extracted from the buffer-insoluble residue of roots with 5% $(NH_4)_2SO_4$ and purified by $(NH_4)_2SO_4$ precipitation, gel filtration on Sepharose 6B and chromatography on N-methylacridinium-Sepharose 4B.

Purified preparations had a specific activity of 210 ± 20 units mg^{-1} protein and contained one active protein and one major inactive protein as determined by polyacrylamide gel electrophoresis and thin layer isoelectric focusing. The AchE had at least 3-fold greater activity against acetylthiocholine than butyl- or propionylthiocholine. The K_m of AchE for acetylthiocholine was 56 µM. The enzyme was stimulated by choline (0.5-50 mM) and totally inhibited by diisopropylfluorophosphate (DIFP, 10^{-4}M) and decamethonium (60 mM). The catalytic center activity determined by DIFP titration was 197 ± 5 mol substrate min^{-1} mol^{-1} active center. The isoelectric point of AchE was 5.3 ± 0.1, the sedimentation coefficient ($S_{20,w}$) was 4.2 ± 0.1 S, and the Stokes radius was 4.00 nm. The mol. wt. calculated from sedimentation and gel filtration data was 76 000 ± 2 000. The mol. wt. determined by SDS-gel electrophoresis was 77 000 ± 2 000. Subunit mol. wts. of 61 000 ± 2 000 (2 x 30 000) and 26 000 ± 2 000 were observed. The enzyme had a frictional ratio (f/fo) of 1.37. A theoretical model

of the quaternary structure of AchE was presented.

Multiple forms of AchE activity were observed following gel filtration in low ionic strength and ion exchange chromatography of preparations having low specific activity. It was suggested that an ionic strength dependent equilibrium existed between aggregates of the 77 000 mol. wt. species. Properties of the bean root AchE were compared with the AchEs from eel and other animal tissues. Though large differences existed in catalytic center activities, substrate hydrolysis rates, and behavior on N-methylacridinium-Sepharose 4B, the AchE from the different sources were similar in many respects.

The specific activity of hypocotyl hook AchE was unaffected by exposure of etiolated seedlings to light or an ethylene source for 3 days. The specific activity of hook AchE increased after 3 days in 10^{-3}M-gibberellin-treated plants and decreased after 4 days in 10^{-4}M-kinetin-treated plants. These results were interpreted in terms of a possible role of AchE in hypocotyl aging.

In a second study, walls of etiolated P. vulgaris hypocotyls were treated with ^{32}P-DIFP under conditions which corrected for adsorption of the radioisotope, to determine the number of nucleophilic sites in cell walls from regions having different elongation rates. Alpha-chymotrypsin and serine were treated similarly to establish optimum conditions for diisopropylphosphorylation. The ^{32}P-phosphoserine content of partially hydrolyzed cell walls was determined. Cell walls from regions of active cell elongation contained 2.71 pmol ^{32}P-phosphoserine mg^{-1} cell wall and those from regions in which cell elongation had

terminated contained no significant ^{32}P-phosphoserine. From these results I concluded that the few nucleophilic sites present were not glycosylation sites in the cell wall but rather were sites in active centers of enzymes which were bound to cell wall preparations.

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CHAPTER I.

STUDIES ON THE ACETYLCHOLINESTERASE OF PHASEOLUS VULGARIS L.

A. INTRODUCTION

Acetylcholine mediates bioelectric responses in a number of animal tissues (Hebb and Krnjveic, 1962; Ruch and Patton, 1965). Regulation of acetylcholine levels is of paramount importance in the control of the information conveyed by this substance. Biosynthesis is completed by the choline acetyltransterase reaction (Nachmansohn and Machado, 1943). Degradation involves either an acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7) or a pseudocholinesterase (acylcholine acylhydrolase EC 3.1.1.8). Acetylcholinesterases (AchEs) are membrane bound enzymes located in nervous tissue, effector organs innervated by cholinergic neurons, and erythrocytes. Their specificity is for the acylcarboxylic acid of the ester (Cohen and Oosterbahn, 1963). Pseudocholinesterases are soluble enzymes located in serum. Their specificity is for the choline of the ester (Augustinsson, 1963). Although it appears that both enzymes are similar in some respects, their tissue localization, substrate specificities, and responses to inhibitors, modulators and excess substrate differ (Rosenberry, 1977). The properties of these enzymes have been reviewed (Froede and Wilson, 1971; O'Brien, 1971; Rosenberry, 1975; Rosenberry, 1977). AchE is involved in regulation of acetylcholine levels *in vivo* (Nachmansohn, 1959). It is inhibited by organophosphates, such as diisopropylfluorophosphate (DIFP), and carbamates, such as neostigmine

or eserine. It is five times more active against acetylcholine than against propionylcholine and at least 100 times more active against acetylcholine than butylcholine. Phenylacetate is hydrolyzed at a rate similar to that for acetylcholine. The enzyme is inhibited by choline and by excess substrate.

Acetylcholine is present in a variety of plant tissues (Fluck and Jaffe, 1974a). Exogenous applications of acetylcholine to mung bean roots have effects similar to those of red light. It inhibits secondary root formation and stimulates H^+ efflux in roots (Jaffe, 1970); it promotes the adhesion of root tips to negatively charged glass surfaces (Tanada, 1972); and it increases oxygen uptake and decreases ATP levels in roots (Junghans and Jaffe, 1972). Applications of acetylcholine promote flowering of Lemna perpusilla (a short day plant) under continuous illumination but prevent flowering of Lemna gibba (a long day plant) under the same conditions (Kandler, 1972), and have the same effect as light in increasing seed germination of at least four species (Holm and Miller, 1972). Both acetylcholine and inductive photoperiods alter the action spectrum of bioelectric responses in spinach (Grerrin, et al., 1973). Red irradiation results in an increase in endogenous acetylcholine levels in mung bean roots (Jaffe, 1970) and other plants (Hartmann and Kilbinger, 1974).

Acetylcholine affects growth responses of wheat seedlings (Dekhuijzen, 1973) and Avena coleoptiles (Evans, 1972), as well as mung bean roots. It inhibits indoleacetic acid (IAA-) induced ethylene production and partially prevents the IAA-promoted delay of hook opening in Phaseolus vulgaris (Parups, 1976) yet mimics the effect of IAA on elongation and peroxidase activity patterns in lentil roots (Penel, et al., 1976).

On the basis of these observations, it may be that acetylcholine mediates the effect of light on bioelectric responses in mung bean roots or other plant tissues through the involvement of phytochrome (Jaffe, 1972).

Few studies of enzymes involved with the regulation of acetylcholine levels in plants have been undertaken. The presence of a choline acetyltransferase has been demonstrated only in one plant species — Urtica dioica (Barlow and Dixon, 1973).

In plant tissues, acetylcholine is hydrolyzed by a variety of esterases including citrus acetylesterase (Jansen, et al., 1947; Schwartz, et al., 1964), wheat germ esterase (Jansen, et al., 1948; Mounter and Mounter, 1962), cucurbitacin esterase (Schwartz, et al., 1964), sinapine esterase (Tsagoloff, 1963), and a cholinesterase (Schwartz, 1967; Riov and Jaffe, 1973; Kasturi and Vasantharajan, 1976). The citrus acetylesterase and wheat germ esterase have K_ms of 1.6 and 1.0 M, respectively, for acetylcholine but have not been tested for DIFP or carbamate inhibition. The cucurbitacin esterase activity is unaffected by 10^{-5}M- DIFP and is slightly stimulated by 10^{-5}M-eserine. The sinapine esterase has a lower (660μM) Km for acetylcholine but is only marginally inhibited by 10^{-3}M-eserine.

However, the cholinesterase, partially purified from mung bean roots and subsequently from a variety of other plant tissues (Riov and Jaffe, 1973; Fluck and Jaffe, 1974d; Kasturi and Vasantharajan, 1976), resembles animal AchE in having inhibition by neostigmine and eserine, maximal activity against acetylesters, a K_m of less than 200μM for acetylcholine or acetylthiocholine, and substrate inhibition.

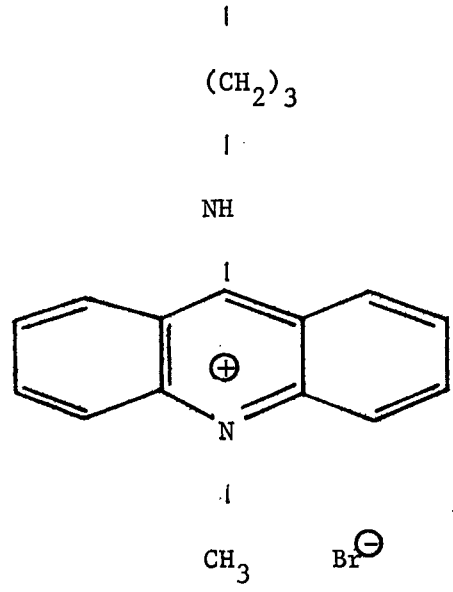
Inhibition of the pea root enzyme by an organophosphate, Fensulfothion, results in an increase in the endogenous acetylcholine levels over control plants with concomitant inhibition of lateral root formation (Kasturi and Vasantharajan, 1976). A rigorous purification of this enzyme has not been achieved, though a variety of chromatographic procedures have been applied (R. A. Fluck, personal communication).

Few AchE assays have been suitable for application to plant tissues or extracts because plants contain substantially lower cholinesterase activities (Fluck and Jaffe, 1974b; Rosenberry, 1977). Even the most sensitive assay which uses the substrate analogue acetylthiocholine (Ellman, et al., 1961) requires an incubation period of several minutes to detect enzyme activity in plant extracts rather than the seconds required in the animal enzyme assay. Spontaneous or non-specific hydrolysis of acetylcholine during the long incubation period is controlled by the addition of 10^{-5}M-neostigmine to assay mixtures (Fluck and Jaffe, 1974b).

Purification of AchE to homogeneity from various animal tissues has involved the use of either the combination of $(NH_4)_2SO_4$ precipitation, gel filtration and ion exchange chromatography in many media (Kremzner and Wilson, 1963; Leuzinger and Baker, 1967) or affinity chromatography (Kalderon, et al., 1970; Berman and Young, 1971; Dudai, et al., 1972a; Rosenberry, et al., 1972). A variety of affinity chromatography matricies have been used to purify eel AchE. The one most suitable for purification of the native molecular form of the enzyme consists of the N-methyl acridinium derivative: 1-methyl-9-[N^{γ}-(ε-aminocaproyl)-γ-aminopropylamino] acridinium bromide hydrobromide; covalently linked to

CNBr-activated Sepharose 4B (MAC-Sepharose 4B) (Dudai, et al., 1972a).

MAC-Sepharose 4B: Sepharose 4B — $NH_2(CH_2)_5$ CONH



This study was undertaken to examine the possibility that an AchE exists which regulates the level of acetylcholine in plant tissues. The objectives of the study were:

(1) To determine the feasibility of using a colorimetric enzyme assay (Ellman, et al., 1961) in which neostigmine incubation acts as a control for spontaneous or non-specific hydrolysis of substrate.

(2) To identify AchE activity in Phaseolus vulgaris L. tissue segments and extracts.

(3) To purify this AchE by chromatography on MAC-Sepharose 4B.

(4) To determine some physical and chemical properties of this enzyme and to compare these properties with those of other AchEs.

(5) To investigate the role of AchE activity in hypocotyl hooks of P. vulgaris.

B. MATERIALS AND METHODS

1. Chemicals

Supplies were obtained from sources as indicated: acrylamide, N, N, N', N'-tetramethylethlylene diamine, and N, N'-methylene bis acrylamide: Eastman Kodak Co., Rochester, N.Y.; trichloracetic acid, etheylene diamine, p-terphenyl, and 1,4-bis-2-(5-phenyloxazolyl)-benzene: Fisher Scientific Co., Pittsburgh, Pa.; isopropanol, dioxane and naphthalene: Mallinckrodt Chemical Works, St. Louis, Mo.; guanidinium chloride and sodium lauryl sulphate (SDS): British Drug House Chemicals, Toronto, Ont.; ^{3}H-DIFP: Amersham/Searle, Arlington Heights, Ill.: DIFP: Aldrich Chemical Co., Milwaukee, Wn.; pHisolytes: Brinkman Instruments, Wesbury, N.Y.; acetylcholine chloride, acetylthiocholine iodide, arginine, bovine serum albumin (BSA), butylcholine chloride, butylthiocholine iodide, catalase, choline chloride, cytochrome c, decamethylenebis-(trimethyl-ammonium bromide) decamethonium bromide), diethylaminoethyl-(DEAE-) Sepharose CL-6B, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), eserine, β-galactosidase, gibberellic acid, glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) (m-hydroxy phenyl)-trimethyl ammonium bromide dimethylcarbamate (neostigmine), kinetin, lysine, myoglobin, ovalbumin and propionylthiocholine iodide: Sigma Chemical Co., St. Louis Mo.; Sepharose 6B, and Sephadex G-75: Pharmacia Fine Chemicals A.B., Uppsala, Sweden; Ethrel: Amehem Products Inc., Ambler, Pa. All other chemicals were obtained locally. "Baker Analyzed" grade (J. T. Baker Chemical Co., Phillipsburg, N.J.) was used when available. Electrophorus electricus AchE and MAC-Sepharose 4B were generously donated by Dr. D. G. Clark, Department of Chemistry, University of British Columbia.

2. Plant Material

Bush bean (Phaseolus vulgaris L. var. Top Crop Green Pod) seeds were surface sterilized with 0.5% (v/v) sodium hypochlorite or 10% (v/v) commercial bleach for 15 min, rinsed four times with water, and grown in vermiculite in plastic trays (McConkey and Co., Sumner, Wn.) for 9 days in a dark cabinet at room temperature (24 ± 2°C). Roots were separated and washed with cold tap water to remove vermiculite. Hypocotyls were separated from cotyledons and rinsed with cold tap water. In one experiment, the hypocotyl was further divided into the regions depicted in Figure 1.

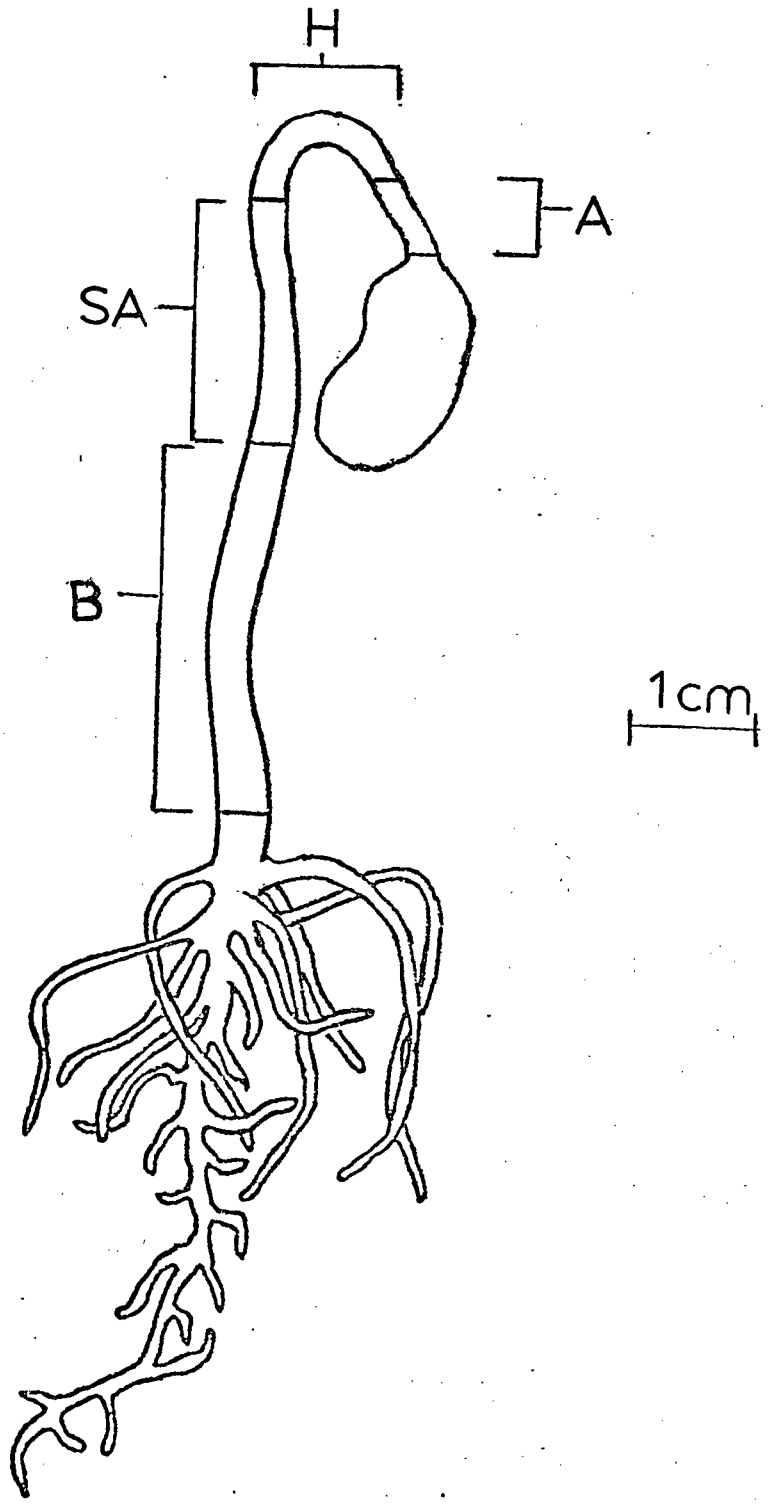
3. Assay of Acetylcholinesterase (AchE) Activity

The activity of AchE was determined by the photometric method of Ellman, et al., (1961) as modified by Riov and Jaffe (1973). Assay mixtures in final volume of 1.62 ml contained 1.00-1.48 ml of 0.5 M-potassium phosphate buffer, pH 8.0, 60 µl of 2.6 mM-DTNB prepared in the same buffer containing 4.5 mM-$NaHCO_3$, and 0.02-0.5 ml of the sample to be assayed. For each sample, a second tube was prepared in which 30 µl of 1.35 mM-neostigmine bromide replaced 30 µl of buffer.

The assay mixtures were incubated for 15 min at 37°C; then 60 µl of 12.5 mM-acetylthiocholine iodide was added. The reaction was allowed to proceed for 10-20 min and was terminated by chilling to 0°C in an ice bath. Absorbance of clear assay mixtures was determined directly at 412 nm.

Particulate assay mixtures were allowed to settle for 10 min and the upper layer was removed with a Pasteur pipette. This suspension was centrifuged at 15,000 g for 15 min and the absorbance of the supernatant was recorded.

Figure 1. Diagram of an etiolated bean seedling showing the regions used in detailed studies of enzyme distribution. A— apical, H — plumular hook, SA — subapical, B — basal.



Enzyme activities were determined by the following calculation:

One unit = $\Delta A_{412} \; v \; t^{-1} \; \varepsilon^{-1}$

where,

ΔA_{412} = A_{412} of the mixture lacking neostigmine minus A_{412} of the mixture containing neostigmine

v = volume of the assay mixture (µl)

t = assay time (min)

ε = extinction coefficient of 2-nitro-5-thiobenzoate (1.36×10^4 nl $nmol^{-1}$)

With the exception of column or gradient effluent fractions, all enzyme assays were performed in duplicate or triplicate.

In the experiment designed to determine the effect of neostigmine on enzyme activity, enzyme solution was boiled for 30 min and cleared by centrifugation at 15 000 g for 10 min. The ΔA_{412} value was the difference between absorbances of corresponding assay mixtures containing boiled and unboiled enzyme solutions.

In experiments designed to examine the effect of a given substance on AchE activity, 30-60 µl of stock solution of the substance was prepared in water (or isopropanol in the case of DIFP) and this replaced the same volume of buffer in the assay mixtures.

The method described by Fluck and Jaffe (1974b) was used for the assay of excised tissue. Assay mixtures having a final volume 20.25 ml contained 18.75 ml of 0.5 M-potassium phosphate buffer, pH 8.0, and 0.75 ml DTNB solution. These were incubated for 15 min at 37°C with 1-3 g of uniformly excised tissue; 0.75 ml of substrate was added and after exactly 8 min, 0.375 ml of neostigmine solution (final concentration of 25 µM) was added. Aliquots (1 ml) were taken at 2 min intervals, the absorbance

was recorded, and the aliquot was poured back into the reaction medium to maintain a constant volume. The absorbances versus time were plotted and the difference between the slopes of the regression lines obtained before and after the addition of neostigmine was used as $\Delta A_{412}\ t^{-1}$ in the calculation of enzyme activity.

4. Protein Determination

The protein in particulate fractions was solubilized by boiling with one volume of 2 M- KOH for 30 min. The solution was diluted ten-fold, shaken, and allowed to settle. The protein in these and all soluble fractions was determined by the method of Lowry, et al., (1951) as modified by Eggstein and Kreutz (1955). The residue of extracted particulate fractions was washed with water and contained no protein detectable by the qualitative microbiuret assay (Goa, 1953).

The protein content of chromatography column and density gradient eluates was estimated by absorbance at 280 nm.

5. Purification of AchE From P. vulgaris

a) Extraction and $(NH_4)_2SO_4$ Precipitation

METHOD A: Roots (344 g) were added to 788 ml of 20 mM-potassium phosphate buffer, pH 7.0, in a Waring blendor and homogenized at 20 000 rpm for 3 min. The homogenate was centrifuged at 4800 g in a PR 6000 centrifuge (International Equipment Co.) for 20 min at -4°C. The supernatant was filtered through Whatman #1 filter paper and the residue was resuspended in 788 ml of the extraction buffer containing 5% (w/v) $(NH_4)_2SO_4$. The slurry was stirred for 30 min at 4°C and centrifuged at 4800 g for 20 min. The supernatant was filtered through two layers of

Whatman #1 filter paper, brought to 80% saturation with solid $(NH_4)_2SO_4$ at 4°C and centrifuged at 4800 g for 20 min. The pellet was resuspended in 30-100 ml of 20 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl and dialyzed overnight against the same buffer at 4°C. The non-diffusible material was clarified by centrifugation at 15 000 g for 10 min. The supernatant could be used immediately or stored at 0°C for up to one month without loss of activity.

METHOD B: Root or hypocotyl tissue (200 g) was extracted with 400 ml of 10 mM-potassium phosphate buffer, pH 7.0, containing 5% (w/v) $(NH_4)_2SO_4$ by the procedures described above. The extract was precipitated in two steps with solid $(NH_4)_2SO_4$ to 40% and 70% saturation. The final pellet was resuspended with 10 mM-potassium phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer, at 4°C. The non-diffusible material was treated the same as in method A. All fractions were dialyzed against buffer before being assayed.

b) Gel Filtration on Sepharose 6B

Extract (5-30 ml) prepared by method A was applied to a 3.5 X 95 cm Sepharose 6B column equilibrated with 20 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl. Gel filtration was performed at a flow rate of 50-60 ml h^{-1}. Fractions were collected and assayed for AchE activity and protein; the active fractions were pooled.

c) Chromatography on MAC-Sepharose 4B

The AchE fraction (87 ml) from gel filtration was applied to a 1.5 X 2.5 cm column of MAC-Sepharose 4B equilibrated with 20 mM-potassium phosphate buffer, pH 7.0, containing 0.2-M NaCl at a flow rate of 6-8 ml h^{-1}; the ligand concentration was 2.0 µmol ml^{-1}. After the entire

sample had entered, the column was washed with approximately 3 column volumes of equilibration buffer and then eluted with approximately 5 column volumes of equilibration buffer containing 1 M-NaCl. Fractions were collected throughout the loading, washing and eluting procedures, and assayed for AchE activity and protein. The total eluted activity was compared with that of the loaded sample to determine the recovery of AchE. The 1 M-NaCl eluate (approximately 30 ml) was concentrated to 2-3 ml by ultrafiltration through an Amicon XM50 membrane filter and stored at $0^{o}C$ for no longer than 12 days. The column was regenerated by washing with approximately 4 bed volumes of equilibration buffer containing 5 M-guanidinium chloride followed by at least 5 bed volumes of equilibration buffer.

6. Labelling With DIFP

METHOD A: A modification of the method of Berman (1973) was used to label specifically AchE purified by MAC-Sepharose 4B. Enzyme solutions (100-400 units ml^{-1}, 185-210 units mg^{-1} protein) in a final volume of 0.810 ml in 20 mM-potassium phosphate buffer, pH 7.0, containing 0.0 or 0.2 M-NaCl were incubated for 15 min at $37^{o}C$ with 15 µl of 52.8 mM-DIFP (in CaO dried isopropanol) or 50 µl of 324 mM-butylcholine chloride, or both DIFP and butylcholine chloride.

The contents of the tubes were dialyzed against 4 changes of the reaction buffer for 18 h at $4^{o}C$. Non-dialyzable mixtures were assayed for AchE activity and protein.

METHOD B: Enzyme solutions (100-400 units ml^{-1}, 185-210 units mg^{-1} protein) in 20 mM-potassium phosphate buffer, pH 7.0, containing 1.0 M-NaCl (final volume = 0.810 ml) were allowed to react for 15 min at $37^{o}C$

in the presence or absence of 50 µl of 1.35 mM-neostigmine bromide. Fifty-six µl of ^{3}H-DIFP (0.26 mg ml^{-1} in propylene glycol, 3.4 Ci mmol^{-1}) were added and the tubes were incubated for 20 min at 37°C. Reaction mixtures were dialyzed against 4 changes of the reaction buffer for 18 h at 4°C; the mixtures were assayed for AchE activity and radioactivity. All procedures were performed on duplicate preparations.

7. Determination of Radioactivity

Ten ml of dioxane based scintillation fluid (Bray, 1960) were added to 20-600 µl samples. Samples were counted at 45% efficiency in an Isocap 300 liquid scintillation spectrometer (Nuclear Chicago) for 2-20 min with an 800 K cpm termination. All activities were below the coincidence counting range of the spectrometer. Background was counted in duplicate before each set of samples and subtracted from cpm values. A quench curve was prepared for activity determinations by the channels ratio method (Wang and Willis, 1965) using ^{3}H-toluene quench standards. In some experiments net cpm values were recorded directly. All samples were counted in duplicate.

8. Polyacrylamide Disc Gel Electrophoresis

Mixtures containing 25-100 µl samples of enzyme purified by either gel filtration or chromatography on MAC-Sepharose 4B (30-150 µg of protein), 5 µl of 0.05% (w/v) bromophenol blue in water, and solid sucrose to 5% (w/v) were applied to 7% (w/v) acrylamide gels. Electrphoresis was performed in small pore gels at pH 8.3 in duplicate on at least duplicate preparations (Davis, 1964). Gels were stained with 1% (w/v) Amido Schwartz in 7% (w/v) acetic acid and destained in 7% acetic acid. The gels were

sliced immediately after electrophoresis and individual 2 mm slices from duplicate gels were assayed for AchE activity in the presence or absence of neostigmine.

9. SDS Gel Electrophoresis

AchE purified by chromatography on MAC-Sepharose 4B and labeled with ^{3}H-DIFP by method B and standard proteins (BSA, β-galactosidase, catalase, G-3-PDH, myoglobin, and ovalbumin) were dialyzed against 10 mM-sodium phosphate buffer, pH 7.2, containing 1% (w/v) SDS for 18 h at room temperature. Samples (75 µl) were mixed with 75 µl of either 10 mM-sodium phosphate buffer, pH 7.2, containing 1% (w/v) SDS, 20% (w/v) sucrose, 0.002 % (w/v) Pyronin Y (a tracking dye), and 40 mM-dithioerythritol or the same solution without 40 mM-dithioerythritol. Mixtures were incubated at 100°C for 5 min, cooled, and 20-100 µl aliquots were applied to 5% (w/v) acrylamide gels (0.8X10 cm) containing 1% (w/v) SDS prepared as described by Weber and Osborne (1969). Electrophoresis was performed at 2 ma per gel for 1 hr and 5 ma per gel for an additional 5.5 h. The center of the tracking dye was marked with India ink. Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in methanol:water:acetic acid (5:5:1, v/v) overnight at 60°C and destained with a 5% (v/v) methanol, 7.5% (v/v) acetic acid solution in water at 60°C for 24 h. Gels were scanned at 550 nm in a Gilford 240 spectrophotometer; absorbance was recorded by a Gilford 6050 chart recorder at 2 cm min^{-1}. Gels were placed on dry ice for 15 min and sliced in 2 mm sections. Slices were placed in scintillation vials containing 0.6 ml of NCS tissue solubilizer (Amersham/Searle):water (9:1,v/v) and kept for 20 h at room temperature followed by 2 h at 50°C. Samples were counted for

radioactivity as described above. Mobilities of all stained and radioactive peaks were determined relative to the mobility of the tracking dye; molecular weights were obtained by comparing the resulting R_f values to a calibration curve prepared from R_f values of standard proteins. All gels were run in duplicate.

10. Sedimentation in Isokinetic Sucrose Gradients

Isokinetic sucrose gradients (10-29.3% (w/v)) were prepared according to the theoretical formulations of Noll(1967) as applied by Morrod (1975). AchE (310 units ml^{-1}, 195 units mg^{-1} protein), or the same preparation labelled with ^{3}H-DIFP by method B, and a mixture of standard proteins (myoglobin, catalase and β-galactosidase) were dialyzed against 20 mM-phosphate buffer, pH 7.0, containing 1.0 M-NaCl. Mixtures containing 175 µl of the active enzyme or 125 µl of the ^{3}H-DIP-enzyme plus 50 µl of the standard proteins mixture, and solid sucrose to 5% (w/v) were applied to the top of the gradient, overlaid with buffer, and centrifuged at 40 000 rpm in a Beckman SW41 rotor for 18 h at 5°C in a Beckman L3-50 ultracentrifuge. Gradients were eluted at a flow rate of approximately 12 ml h^{-1}; 0.5 ml fractions were collected and assayed for AchE activity or radioactivity. Catalase and myoglobin were located by measuring the absorbance at 405 nm. β-galactosidase was assayed as described by Massoulie and Rieger (1969). A calibration curve was prepared for each gradient using the standard proteins. All sedimentation experiments were run in duplicate.

11. Isoelectric Focusing

Isoelectric focusing was performed by the thin layer method (Radola, 1973). Sephadex G-75 (7.0 g) was swollen in 100 ml of deionized water

over boiling water for 1 h. Four ml of pHisolytes and 0.1 g each of lysine and arginine were added to the cooled suspension. Glass plates (20X20 cm) were prepared as described by Radola (1973).

Samples were dialyzed against 10 mM-potassium phosphate buffer, pH 7.0, overnight at 4°C. These and standard proteins — BSA, cytochrome c and myoglobin — (10 mg ml^{-1}) were applied individually in 18 mm bands by diffusion from a coverslip. One or more applications of 20-50 µl samples totalling 100-200 µg of protein were made 7-10 cm from the cathode end of the plate. Samples focussed in 10 mm thick dextran were applied by replacing a 1 cm band of dextran with similarly prepared dextran containing up to 1 ml of sample. Twenty µl of each standard were applied 1-3 cm from both the cathode and anode end of the plate. Plates were placed in the TLE double chamber (Desaga). The platinum ribbon electrodes were put on thin strips of Whatman #1 paper moistened with 0.2 M-H_2SO_4 (anode) or 0.4 M-ethylenediamine (cathode). Focusing was carried out at 4°C at 200 V for 8 h and 500 V for an additional 10 h at which time colored standard proteins were focused.

Measurements of the pH were made directly with a flat membrane glass electrode (Desaga) or 0.3 cm bands were removed from the dextran layer, diluted with 200 µl of deionized water, and measured. Protein was detected by the paper print method of Delincee and Radola (1972) using Coomassie Brilliant Blue R-250 or bromophenol blue. Activity of AchE was detected by assaying samples (appröximately 100 µl) which were removed from the dextran layer. This method proved adequate for qualitative purposes but quantitative estimation was not achieved. In two experiments, dextran layers removed from the plate were eluted from either a 25 ml syringe

containing glass wool or a 2.5 X 24 cm column of Sephadex G-25 with 10 mM-potassium phosphate buffer, pH 7.0. All isoelectric focusing experiments were duplicated.

Isoelectric focusing was also performed in a sucrose density gradient (0-64% (w/v)) containing 1% (w/v) ampholytes in a 110 ml column (LKB) as described by Morrod (1975). The linear gradient was prepared by mixing two solutions, one solution containing 1.87 ml of Ampholines (LKB), 28 g of sucrose and 42 ml of water and another solution containing 0.63 ml of Ampholines, 1.5 ml of enzyme solution (251 units ml^{-1}), 204 units mg^{-1} protein) in 10 mM-potassium phosphate buffer, pH 7.0 and 53.87 ml of water. Focusing was carried out at 3°C for 44 h: at 300V for 5 h, at 700V for 18 h, at 1250V for 6 h, and at 1500V for 15 h. The column was eluted at 2.5 ml min^{-1} and 1 ml fractions were assayed for AchE activity, protein, and pH.

12. Determination of Stokes Radius and Molecular Weight

A Sepharose 6B column (0.8 X 70 cm) was equilibrated with 20 mM-potassium phosphate buffer, pH 7.0, containing 1.0 M-NaCl. A solution containing Blue Dextran 2000 and $K_3Fe(CN)_6$ (2 mg ml^{-1} each) in equilibration buffer was applied at a flow rate of 5 ml hr^{-1}. Fractions (0.8 ml) were collected and absorbance at 650 nm and 410 nm was recorded to determine the void and total volumes, respectively. In a second run, standard proteins (β-galactosidase (6.9 nm), catalase (5.2 nm), and myoglobin (1.9 nm) — 2 mg ml^{-1} each) were applied under the same conditions. Catalase and myoglobin were detected by absorbance at 405 nm and β-galactosidase was assayed as described by Massoulie and Rieger (1969). The partition coefficient (K_{av}) was determined as described by Pharmacia Fine Chemicals and a calibration curve was prepared by plotting the Stokes radii (R_e) of

standard proteins vs $\sqrt{-\log K_{av}}$. The K_{av} of AchE was determined by the preparative gel filtration described above. The Stokes radius of AchE was obtained from the calibration curve and molecular weight was determined by the combined Svedberg equation and Stokes-Einstein equation (Pang, 1975):

$$M = S R_e 6N\pi\eta(1-\bar{v}\rho)^{-1}$$

where,

S = sedimentation coefficient x 10^{13}

R_e = Stokes radius

η = 0.01002 poise (the viscosity of water at 20 C)

$\bar{v}_\rho$ = 0.75 $cm^3 g^{-1}$ (partial specific volume of eel AchE reported by Bon, et al. (1973))

ρ = 1.00 g cm^{-3}

The frictional ratio (f/fo)was calculated from the equation (Seigel and Monty, 1966):

$$\frac{f}{fo} = \frac{Re}{(3\bar{v}\, M/\, 4\pi N)^{1/3}}$$

13. Ion Exchange Chromatography on Deae-Sepharose CL-6B

A 10 ml sample (45 units ml^{-1}) prepared by extraction method B was applied to a column (2.8 X 57 cm) of DEAE-Sepharose CL-6B equilibrated with 20 mM-potassium phosphate buffer containing 0.03 M-NaCl and 0.01% (w/v) NaN_3, pH 7.0, at a flow rate of 15 ml h^{-1}. The column was eluted with 315 ml of buffer and a NaCl gradient (0.03-0.8 M). Fractions (7 ml) were collected and assayed for AchE activity and protein.

The method of affinity elution (Scopes, 1977) was performed in 20 mM-potassium phosphate buffer, pH 7.2. A 10 ml sample (45 units ml^{-1}) prepared by extraction method A was applied to a column (5.5 X 10 cm) of

DEAE-Sepharose CL-6B equilibrated with buffer, at a flow rate of 45 ml h^{-1}. The column was eluted with 170 ml of buffer containing 1 mM-guanidinium chloride, and 170 ml of buffer containing 1 mM-acetylcholine. Fractions (9 ml) were collected and assayed for AchE activity and protein.

14. Cell Wall Extraction

Hypocotyl tissue (50 g) was ground to a fine powder in liquid nitrogen (8-10 min). The frozen powder was left to melt at 0°C. The following extraction was carried out at 0-4°C. The homogenate was suspended in 500 ml of 10 mM-potassium phosphate buffer, pH 7.0, and allowed to settle until 2 layers appeared (10-20 min). The upper layer was transferred to centrifuge bottles. The lower layer was resuspended and the settling procedure was repeated twice. The cell wall fragments were collected from the pooled upper layers by centrifugation at 15 000 g for 10 min. The pellet was resuspended in 200 ml of buffer. This suspension contained less than 2% of the cell fragments as intact cells (determined by light microscopy). The suspension was treated with a Blackstone Ultrasonic Probe for 2 min at 200 ± 50 W to release attached cytoplasmic contaminants. The fragments were collected by centrifugation at 10,000 g for 15 min, resuspended in buffer and treated again with the ultrasonic probe. This procedure was repeated until the cell walls were free from cytoplasmic contaminants as determined by phase contrast microscopy. Four washings were usually sufficient to achieve the desired purity. The cell wall suspension was assayed for AchE activity and protein. Assays were performed in duplicate or triplicate on two cell wall preparations.

15. Growth Regulator Experiments

The following procedures were performed to determine whether a range of exogenous stimuli affect AchE activity in hypocotyl hooks of etiolated seedlings. Surface sterilized beans were grown for 5 days in darkness in plastic trays (2 per treatment). The etiolated seedlings were sprayed with approximately 10 ml of one of the following: 10^{-3} M-gibberellin, 10^{-4} M-kinetin, 100 ppm 2-chlorosulphonic acid (as Ethrel, an ethylene source) or water. One tray of seedlings was exposed to fluorescent light (cool white, 2×10^{3} erg cm^{-2} sec^{-1}). A sample (2-4 g) of hypocotyl hooks was harvested daily from each flat and the spray treatment was repeated. These operations were performed in dim green light (40 W filtered by a Carolina Biological Supply (CBS) GR 545 filter). Hooks were frozen in liquid nitrogen and ground to a fine powder in a mortar. Two volumes of 10 mM-phosphate buffer, pH 7.0, were added and the suspensions thawed in an ice bath and dialyzed for 18 h against buffer at 4°C. Samples (500 µl) were assayed for AchE activity and protein. Assays were performed in duplicate or triplicate on three separate preparations.

16. Hypocotyl Hook Angle Measurements

Nine-day-old etiolated beans were harvested in dim green light (25 W incandescent light filtered by a CBS GR 545 filter) and hooks were excised as described by Klein, et al. (1956) and incubated in petri plates containing 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} M-acetylcholine chloride in water for 24 h in darkness. One group containing no acetylcholine chloride was exposed to red light from a 25 W incandescent bulb filtered by a CBS red 650 filter. Hook angles were determined by the method of Klein, et al. (1956). The experiments

were performed in duplicate.

17. Elongation Measurements

Seven-day-old etiolated bean plants were sprayed with 10^{-6} M-acetylcholine chloride in water or water alone, measured in dim green light (25 W incandescent light filtered by a CBS GR 545 filter) and measured again after 24 h. Measurements were made from the base of the hypocotyl along the length of the hypocotyl to the cotyledonary node using a flexible ruler.

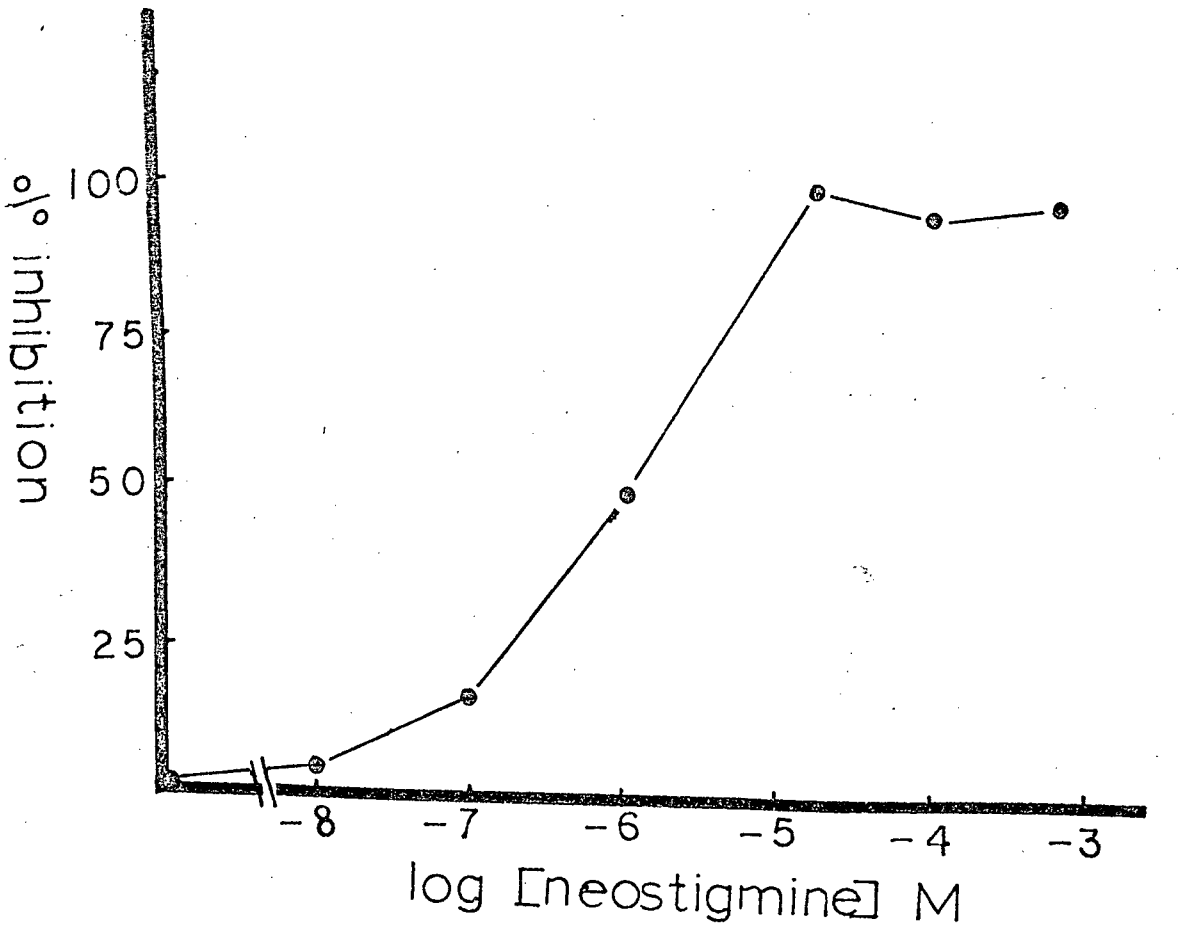
C. RESULTS

1. The Assay Methods

a) The effect of neostigmine on AchE activity

The assumption that neostigmine-inhibitable hydrolysis of acetylthiocholine was identical with AchE activity was tested by determining the effect of neostigmine on the hydrolysis of acetylthiocholine. The results are shown in Figure 2. Concentrations of neostigmine greater than 10 μM inhibited substrate hydrolysis by 90% in preparations having a specific activity of 12 units mg^{-1} protein. The curve was characteristic of neostigmine inhibition of AchEs (Karczmar, 1967; Riov and Jaffe, 1973) and validated the assumption that neostigmine-inhibitable hydrolysis of acetylthiocholine was identical with AchE activity in bean root preparations. Neostigmine-inhibited preparations remained inactive after dialysis against 3 changes of buffer for 36 hr at $4^{o}C$. All esterase activity in preparations having a specific activity greater than 20 units mg^{-1} protein was neostigmine inhibitable.

Figure 2. The effect of neostigmine on AchE activity. Root extracts were precipitated with $(NH_4)_2SO_4$ by method A. Values are means of two assays from a duplicated experiment.



b) The effect of assay time on AchE activity

The neostigmine-inhibitable hydrolysis of acetylthiocholine was time dependent (Figure 3). The absolute ΔA_{412} values and the slope varied depending on the preparation used, however linearity from 3-18 min was reproducible for soluble and particulate fractions of both root and hypocotyl preparations. Extrapolation of the line to t=0 yielded a positive ΔA_{412} value, which reflected the time lag between termination of the assay incubation and the measurement of absorbance. The assay was suitable for use when longer incubation times were needed to detect low enzyme activities.

c) The effect of enzyme quantity on AchE activity

The activity of AchE depended directly on the quantity of solution assayed (Figure 4). The relationship was linear for ΔA_{412} values ranging from 0.02 to approximately 0.85 (for the experiment presented in Figure 4 this corresponds to 5-100 µl). The regression coefficient was 0.996.

2. Extraction and $(NH_4)_2SO_4$ Precipitation of AchE From P. vulgaris Roots and Hypocotyls

The crude preparations which were obtained after homogenization of either root or hypocotyl tissues in 10 mM-potassium phosphate buffer, pH 7.0, had both low AchE activity and low specific activity (Table I). The activities were increased 7 fold in roots and 20 fold in hypocotyls after these preparations were dialyzed against 4 l of deionized water and then 2 changes of 4 l of 10 mM-potassium phosphate buffer, pH 7.0, for 36 h at 4°C. The activity of these preparations was reduced when the diffusate from either root or hypocotyl dialysis was

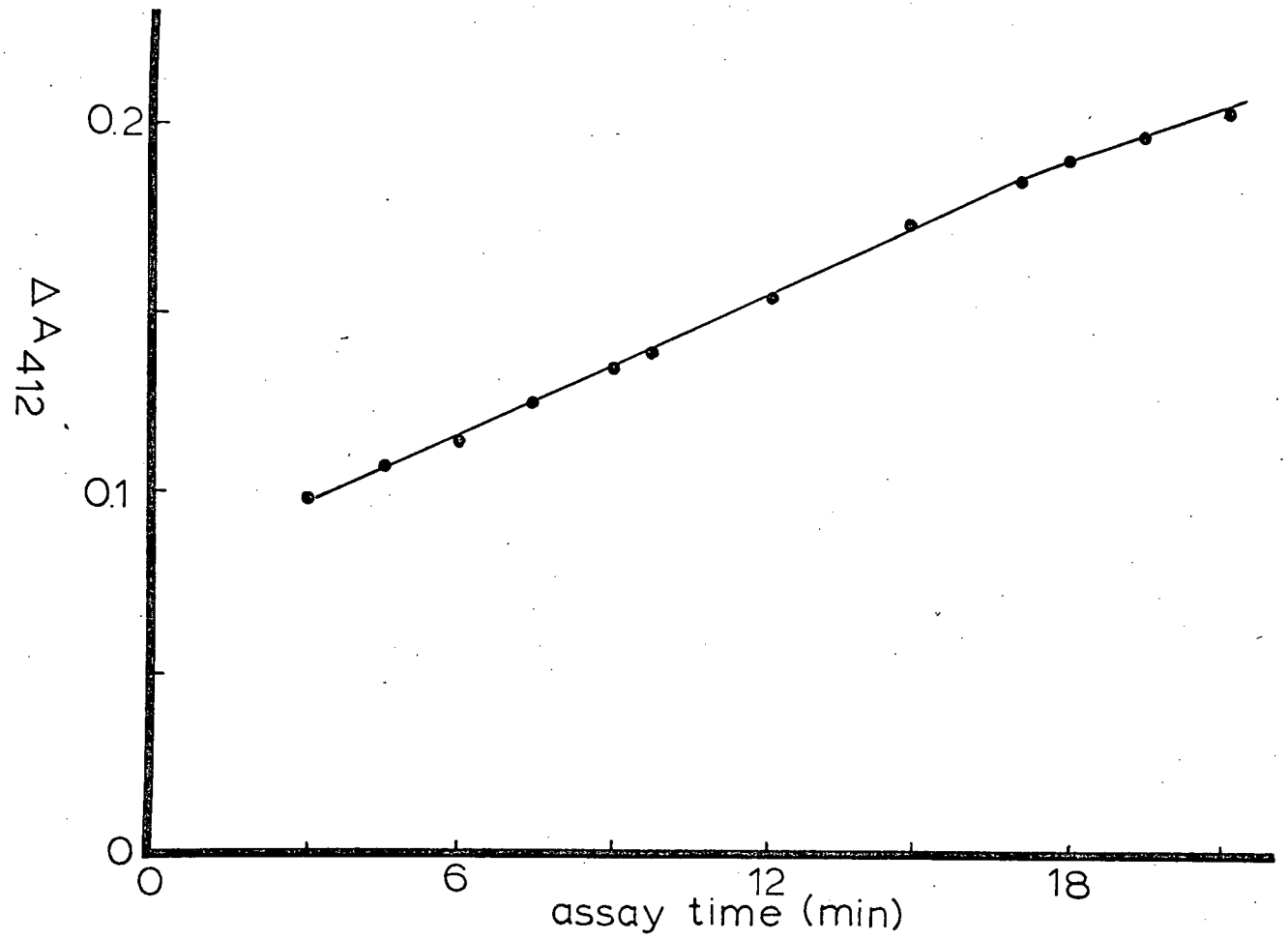


Figure 3. Neostigmine-inhibitable hydrolysis of acetylthiocholine as a function of assay time. Values are means of two assays of a preparation containing 11.5 units mg^{-1} protein.

Figure 4. The effect of enzyme quantity on the AchE activity. Values are means of two assays of a preparation containing 11.9 units mg^{-1} protein.

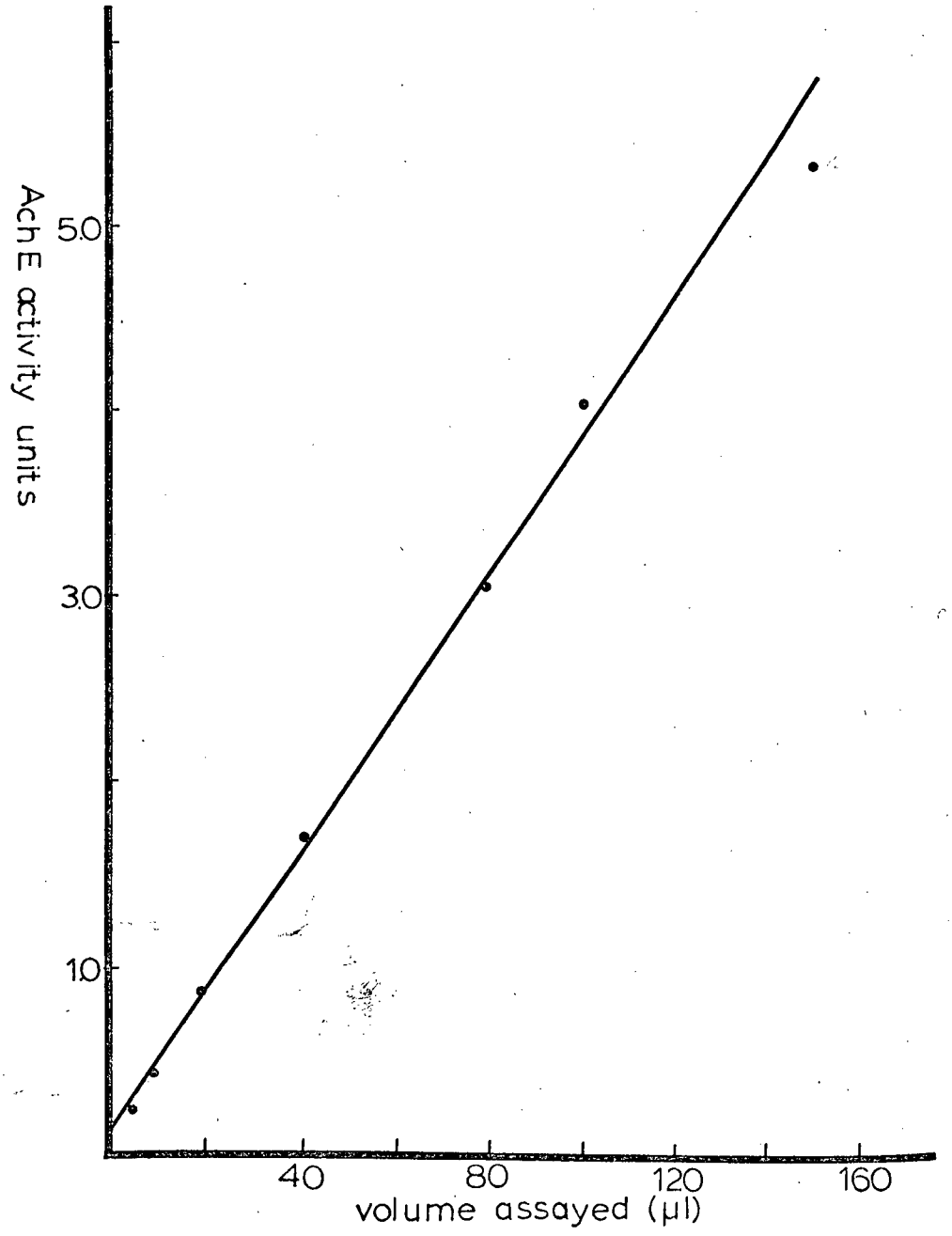


Table I: The effect of dialysis and the reintroduction of the diffusate to the non-dialyzable homogenate on the AchE activity in P. vulgaris root and hypocotyl. Each tissue was homogenized in 10 mM-potassium phosphate buffer, pH 7.0, and immediately dialyzed against 4 1 of deionized water, and then buffer. The diffusate in water was reduced to the original sample volume and returned to the non-dialyzable fraction. N.D. denotes values not determined.

Fraction Assayed	Origin of Diffusate	Activity (g^{-1})*	Sp. Activity (mg^{-1})**	% of Initial Activity	% Decrease of Activity in Non-Dialyzable Substances
Tissue Origin: ROOT					
Homogenate Before Dialysis		3.15	0.25	100	---
Homogenate After Dialysis		20.4	2.83	648	0
Dialyzed Homogenate After Addition of Diffusate	Root	15.2	N.D.	482	25
	Hypocotyl	8.5	N.D.	270	58
Tissue Origin: HYPOCOTYL					
Homogenate Before Dialysis		0.20	0.02	100	---
Homogenate After Dialysis		3.9	0.47	1950	0
Dialyzed Homogenate After Addition of Diffusate	Root	2.2	N.D.	1100	43
	Hypocotyl	1.3	N.D.	650	67

* weights are given as fresh weight
** weights are given as Lowry protein

reintroduced to either root or hypocotyl preparations. However, this activity loss did not fully correspond to the increased activity produced by the dialysis.

A rapid extraction method (method B) in which the extraction buffer contained 5% $(NH_4)_2SO_4$ was tested on roots and hypocotyls. The results are shown in Table II. Although the 5% $(NH_4)_2SO_4$ extraction procedure was more efficient for hypocotyls, in which 81% of the total activity was extracted, than for roots, in which 43% of the total activity was extracted, subsequent $(NH_4)_2SO_4$ precipitation resulted in higher activities on protein and fresh weight bases in roots than in hypocotyls. Thus, further purification efforts were directed toward the root AchE.

3. Localization of AchE in P. vulgaris

a) AchE activity in excised pieces of root and hypocotyl

The AchE activity of excised portions of etiolated bean seedlings is shown in Table III. Activity S.E.M. values were very large because small absorbance changes were recorded. Enzyme activity expressed on both a fresh weight and protein basis was highest in roots and lowest in the basal regions of hypocotyls, but increased with distance up the hypocotyl. Activity of entire hypocotyls was estimated at 0.1 units g^{-1} fresh weight of hypocotyl. This value was based on the observation that the fresh weight of subapical and basal segments comprised greater than 80% of the fresh weight of hypocotyls. Both the hypocotyl and root activities were lower than corresponding values obtained by assaying tissue homogenates (Table I).

Table II: Recovery of AchE from P. vulgaris roots and hypocotyls extracted and fractionated with $(NH_4)_2SO_4$. Values are averages of at least three preparations (method B). N.D. denotes values not determined.

FRACTION		ROOT		HYPOCOTYL	
		Activity (g^{-1} units)*	Sp. Activity (mg^{-1} units)**	Activity (g^{-1} units)*	Sp. Activity (mg^{-1} units)**
5% (w/v) $(NH_4)_2SO_4$	residue	8.2	N.D.	0.06	N.D.
	extract	6.1	3.2	0.25	0.2
10-40% Saturation	precipitate	0.2	N.D.	0.06	N.D.
	supernatant	5.8	3.8	0.21	1.1
40-70% Saturation	precipitate	5.0	7.1	0.21	1.0
	supernatant	0.9	N.D.	0.00	N.D.

* weights are given as fresh weight
** weights are given as Lowry protein

Table III: Activity of AchE in excised segments of roots and regions of the hypocotyls of P. vulgaris shown in Figure 1. Intact 12 cm segments of tissue were assayed as described in Materials and Methods. AchE activity values are means of four determinations ± S.E.M. Protein values are means of 3 assays on at least duplicate samples ± S.E.M.

Origin of Excised Segments	Activity (units mg^{-1} fresh wt.$^{-1}$)	Total Protein (mg g^{-1} fresh wt.)	Specific Activity (units mg^{-1} protein)
Apical Region of Hypocotyl	0.370 ± 0.199	16.8 ± 0.8	0.022
Hook Region of Hypocotyl	0.206 ± 0.066	15.3 ± 0.6	0.013
Subapical Region of Hypocotyl	0.087 ± 0.024	9.7 ± 0.7	0.009
Basal Region of Hypocotyl	0.072 ± 0.038	8.7 ± 0.5	0.008
Roots	0.700 ± 0.140	7.3 ± 0.2	0.096

b) Activity of AchE in the cell wall

The hypocotyl cell walls prepared by successive washes with buffer were assayed for AchE activity by the particulate assay procedure. The activity was 0.033 ± 0.005 units mg^{-1} dry cell wall, and the specific activity of these preparations was 0.25 ± 0.04 units mg^{-1} cell wall protein.

4. Purification of Root AchE

Low activities were detected in extracts of roots homogenized in either 10 or 20 mM-potassium phosphate buffer, pH 7.0, after centrifugation and filtration (Table IV). Values for activity in crude extracts were close to the detection limits of the assay procedure and consequently varied from one experiment to the next. These were consistently less than 5% of the total activity in homogenates (Tables I and II). The results presented in Table IV were obtained when the resulting residue was extracted with buffer containing 5% (w/v) $(NH_4)_2SO_4$ and partially purified by $(NH_4)_2SO_4$ fractionation, gel filtration on Sepharose 6B, and chromatography on MAC-Sepharose 4B. Specific activity after $(NH_4)_2SO_4$ fractionation was greater than that obtained after extraction by method B (Table II), although total recovery was 5.0 units g^{-1} fresh weight in both cases. The elution profile shown in Figure 5 was obtained when AchE was chromatographed on Sepharose 6B. AchE activity eluted as a single peak having a K_{av} of 0.647.

The elution profile shown in Figure 6 was obtained when AchE prepared by gel filtration on Sepharose 6B was chromatographed on the MAC-Sepharose 4B. Specific activity of the concentrated 1 M-NaCl

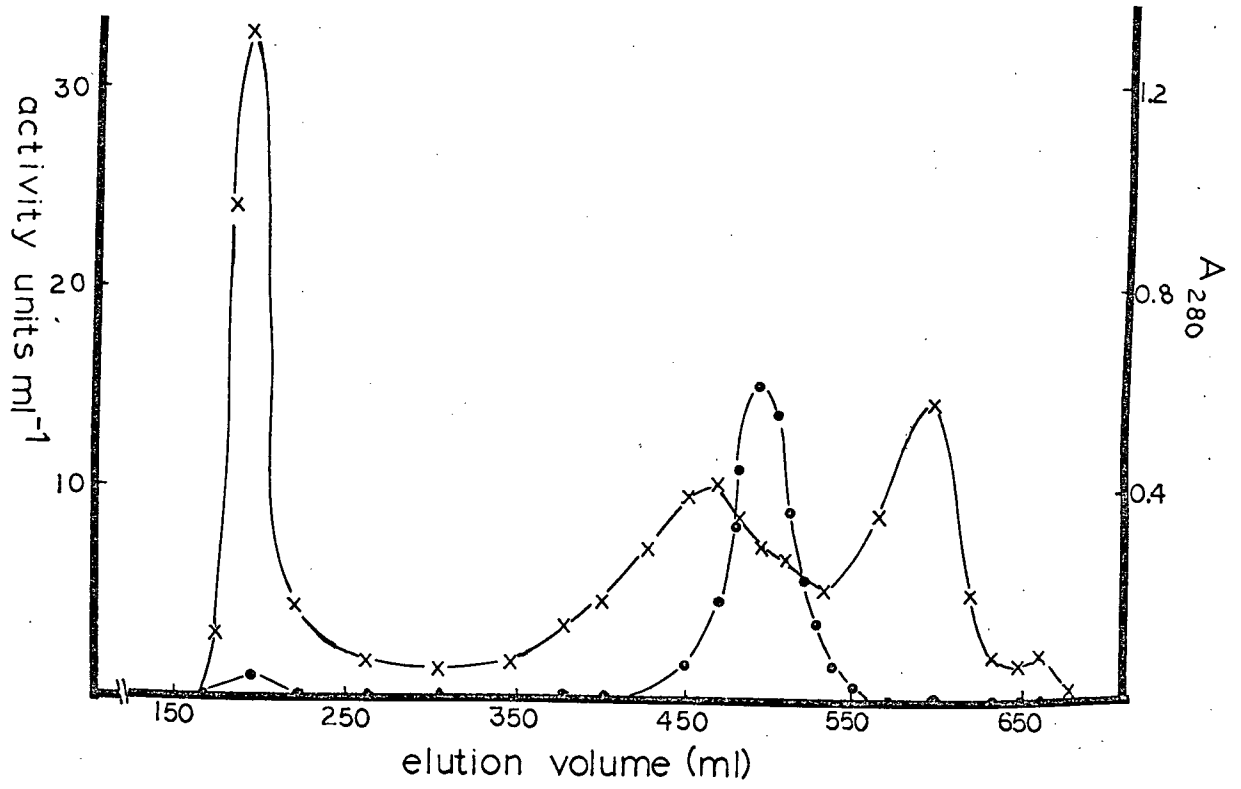


Figure 5. Elution profile obtained after gel filtration of AchE on Sepharose 6B. Samples were prepared by method A. AchE activity (● — ●) and A_{280} (x —— x) of each 10 ml fraction were measured.

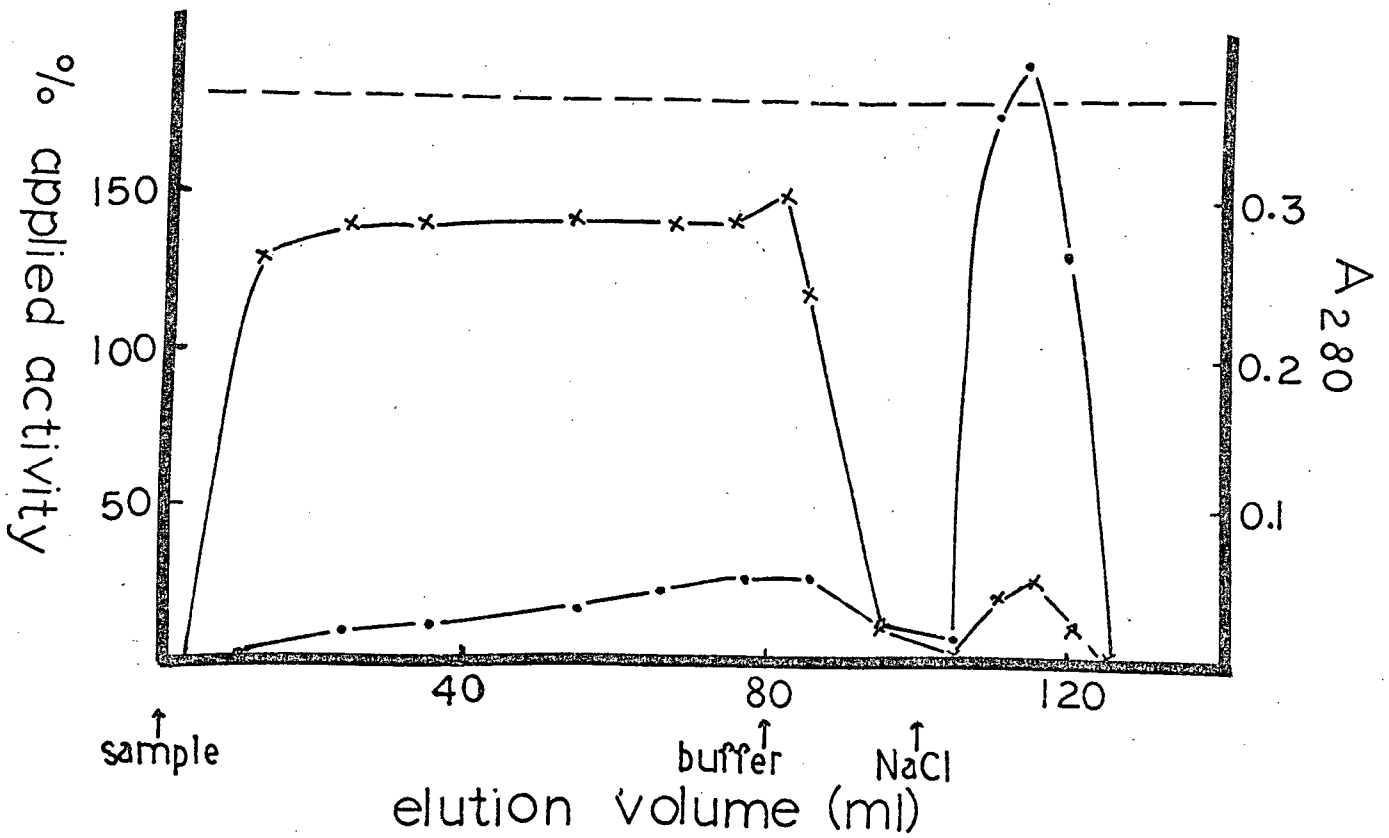


Figure 6. Elution profile obtained after chromatography of AchE on MAC-Sepharose-4B containing 2.0 μmol of ligand ml^{-1}. The sample obtained from gel filtration was loaded in 20 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl. The column was washed with the buffer then with the buffer containing 1.0 M-NaCl. AchE activity (● ——○●) and A_{280} (x —— x) of each fraction were measured. The horizontal dashed line indicates the A_{280} of the sample (0.365).

Table IV: Purification of AchE from P. vulgaris roots. Methods of extraction and purification are detailed in Materials and Methods. Values presented were obtained from one preparation and are representative of at least two other preparations. Purification values are based on the specific activity of dialyzed root homogenates from Table I.

Fraction	Volume (ml)	Protein (mg)	Total Units (units)	Specific Activity ($units \cdot mg^{-1}$ protein)	Recovery (% of 5% $(NH_4)_2SO_4$ Extract)	Purification (-fold)
Crude Extract (in buffer)	848	N.D.	508	N.D.	---	---
5% $(NH_4)_2SO_4$ Extract of Residue	695	662.3	4239	6.4	100	2.3
80% $(NH_4)_2SO_4$ ppt. (resuspended)	30	149.2	1775	11.9	42	4.2
Sepharose 6B	87	74.2	1722	23.2	41	8.2
MAC-Sepharose 4B (after ultrafiltration)	2.9	2.6	589	222.9	14	78.8

eluate varied from 190-230 units mg^{-1} protein.

5. Characterization of Purified AchE

a) DIFP Labeling

Figure 7 shows that AchE activity was completely inhibited at DIFP concentrations greater than 10^{-4}M. The inhibition of enzyme activity by DIFP was reduced from 99% to 29% in the presence of 20 mM-butylcholine in 20 mM-phosphate buffer, pH 7.0, and from 94% to 63% in the presence of 200 mM-butylcholine in 20 mM-phosphate buffer, pH 7.0, containing 0.2 M-NaCl. The latter treatment completely protects animal AchEs from DIFP inactivation (Cohen, et al., 1967). The incomplete protection against DIFP inhibition that butylcholine provided to the bean enzyme prevented the use of butylcholine in experiments designed to specifically label AchE with radioactive DIFP.

Table V shows that there was greater incorporation of ^{3}H-DIFP into preparations labeled in the absence rather than in the presence of 125 μM-neostigmine. The catalytic center activity, based on DIFP binding sites, was 606 and 197 ± 5 mol. of substrate min^{-1} mol^{-1} DIFP binding site when corrected and uncorrected, respectively, for binding in the presence of neostigmine. The corrected value was based on the assumption that neostigmine completely inhibited DIFP binding to AchE.

b) Disc Gel Electrophoresis

Results of polyacrylamide gel electrophoresis of AchE preparations from two stages of purification are shown in Figure 8. There were two densely stained bands of protein (R_f=0.07±0.03 and R_f=0.69±0.03) and a lightly stained region (from R_f=0.39-0.49) after gel electrophoresis of high specific activity (195-229 units mg^{-1} protein) preparations. A

Figure 7. The effect of DIFP on the AchE activity purified by chromatography on MAC-Sepharose 4B. Values are means of two assays performed on a preparation containing 96 units ml^{-1} and 229 units mg^{-1} protein.

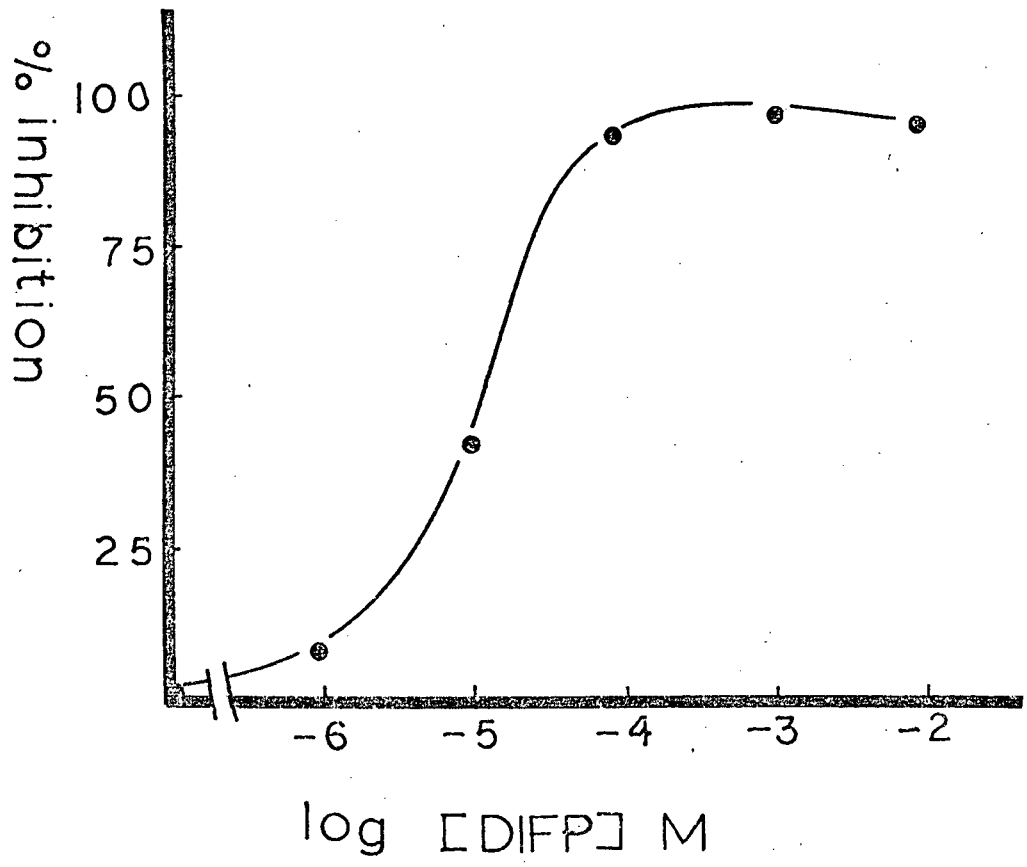


Figure 8. Polyacrylamide gel electrophoresis of AchE. Samples were prepared by either a) gel filtration on Sepharose 6B, or b) chromatography on MAC-Sepharose 4B and separated by electrophoresis in 7% (w/v) polyacrylamide gels at pH 8.3. Gels were stained with 1% (w/v) Amido Schwartz (a1 and b1) or cut into 2 mm slices and assayed for AchE activity (b2). Protein zymograms are based on R_f values averaged from duplicate gels of two (a1) or three (b1) different preparations. The enzyme activity profile shows values from one of two duplicate experiments.

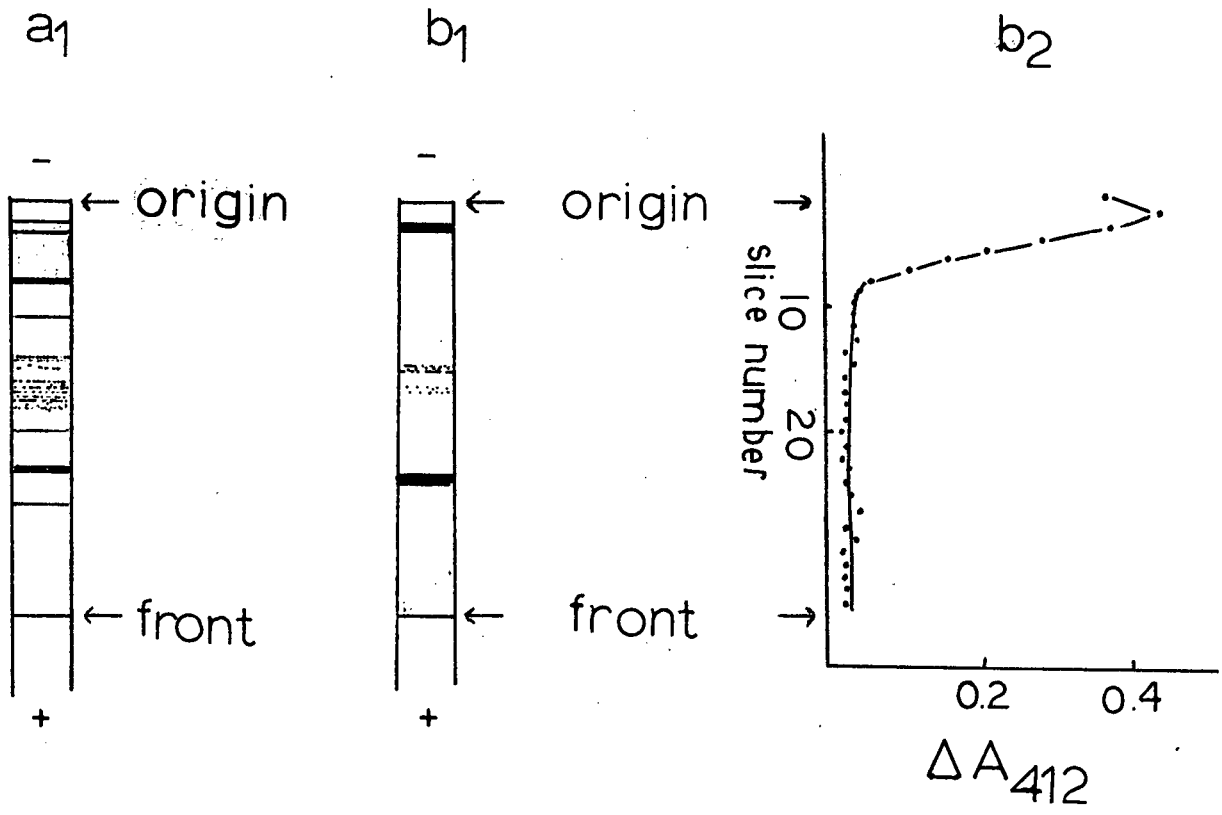


Table V: Incorporation of ^{3}H-DIFP into AchE purified by chromatography on MAC-Sepharose 4B. Preparations containing 100-300 units ml^{-1} and 185-210 units mg^{-1} protein were reacted with 10^{-4} M-^{3}H-DIFP in the presence or absence of 125 μM-neostigmine and counted for radioactivity after exhaustive dialysis. Estimated catalytic center activity was based on DIFP binding sites. Values are means of three samples of duplicate preparations ± S.E.M. N.D. denotes value not determined.

	^{3}H-DIFP incorporated (pmol $unit^{-1}$)	Estimated catalytic center activity (mol of substrate min^{-1} mol^{-1} DIFP)
No neostigmine	5.07±0.12	197±5
Neostigmine	3.42±0.15	N.D.
Net value	1.65	606

single peak of AchE activity was located (R_f=0.05) that corresponded with one of the bands of protein. This peak accounted for more than 90% of the applied activity. Preparations having lower specific activity (21-30 units mg^{-1} protein) showed additional protein bands.

The methods of Koelle (1951) as modified by Wright and Plummer (1973) and Karnovsky and Roots (1964) were applied without success to detect AchE activity in polyacrylamide gels.

c) SDS Gel Electrophoresis

Figure 9 shows the calibration curve obtained after SDS polyacrylamide gel electrophoresis of standard proteins. AchE was purified by chromatography on MAC-Sepharose 4B and labeled with ^{3}H-DIFP. The scans shown in Figures 10a and 11a were obtained after SDS gel electrophoresis of this preparation under non-reducing and reducing conditions, respectively. Figures 10b and 11b show the radioactivity in slices of the same gels. The major DIFP-labeled regions corresponded to mol. wts. of 61 000 ± 2 000, 26 000 ± 1000 and 17 500 ± 500. A minor DIFP-labeled peak had a mol. wt. of 77 000 ± 2000. The 77 000, 61 000, and 26 000 DIFP-labeled components corresponded to distinct protein peaks in the gel scans. Additional low molecular weight (16 000) protein was observed in the gel scans but no corresponding tritiated peak was seen. The gel scan of reduced AchE showed the same protein peaks. There was less radioactivity in the tritiated-61 000 mol. wt. subunit and an increase in activity in a 30 000 ± 1000 mol. wt. component. Complete reduction of the 61 000 mol. wt. subunit was not observed. The same results were obtained for AchE labeled with DIFP in the presence of neostigmine.

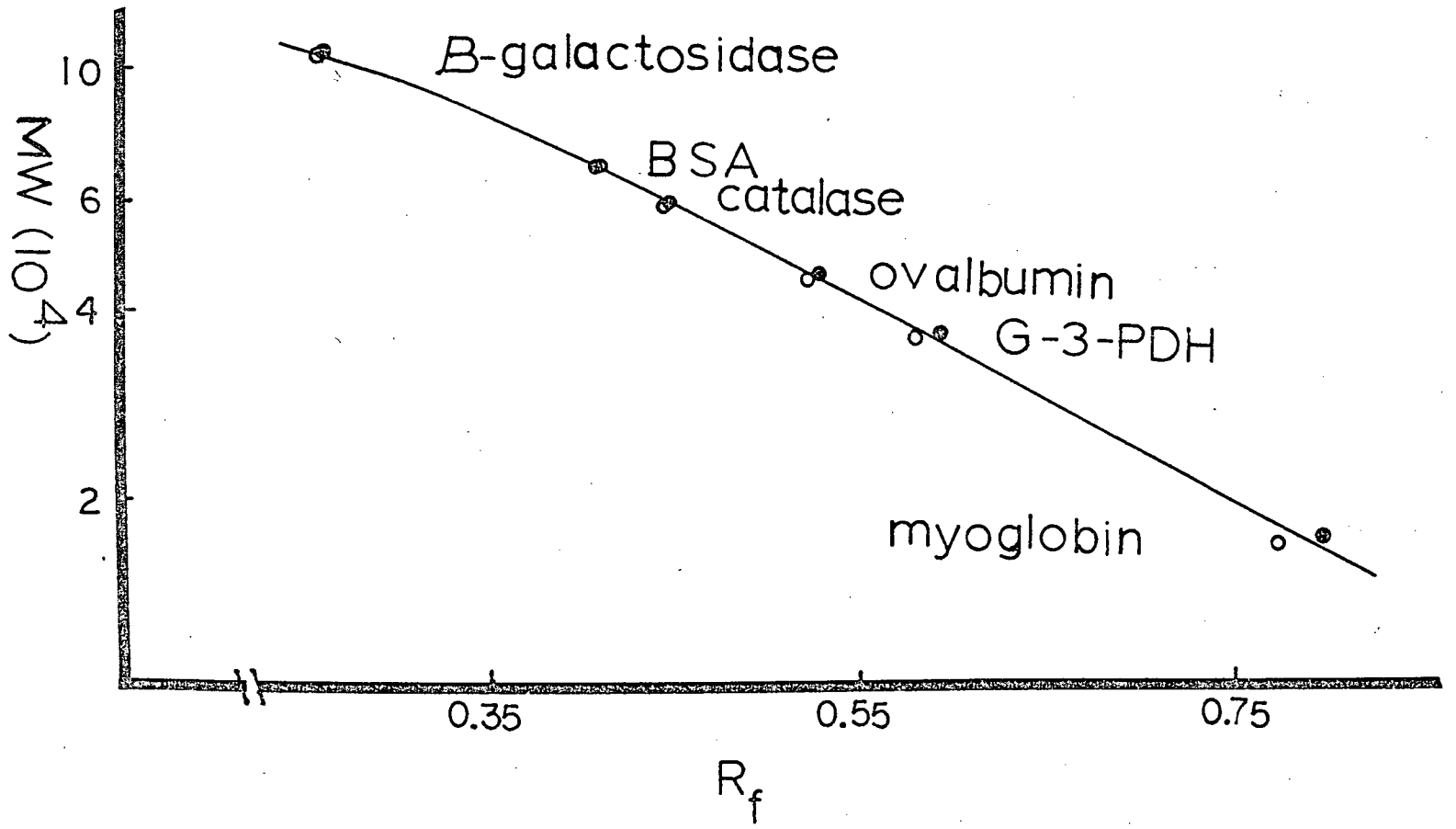


Figure 9. Mobility of standard proteins relative to the tracking dye in polyacrylamide gels containing 1% SDS. Values obtained from duplicate gels are presented.

Figure 10. Distribution of (a) protein and (b) tritium after SDS-acrylamide gel electrophoresis of AchE purified by chromatography on MAC-Sepharose 4B and labeled with ^{3}H-DIFP. AchE was denatured in the absence of any reducing agents. Gels were stained with Coomassie Blue, scanned for absorbance at 550 nm, sliced into sections, and assayed for radioactivity. Molecular weights are averages from duplicate gels.

Figure 10a

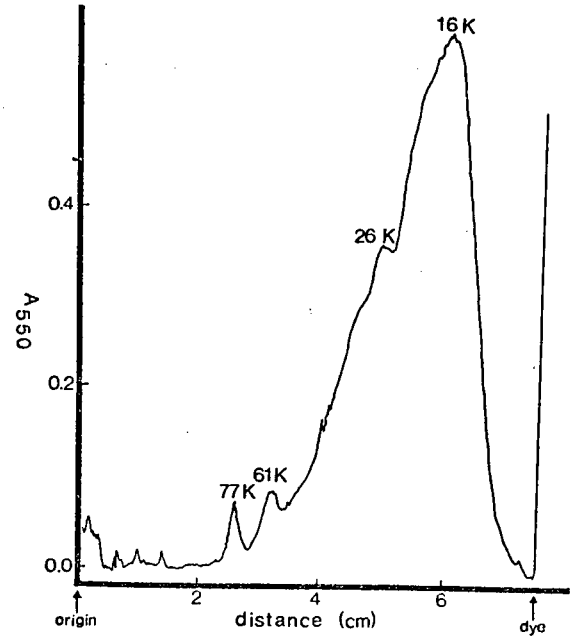


Figure 10b

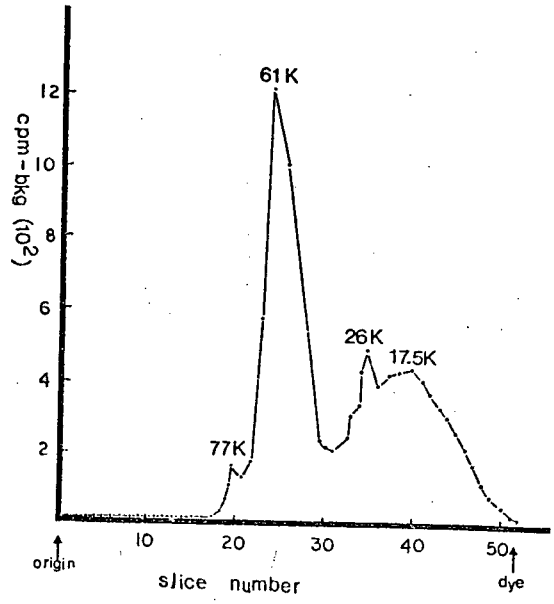


Figure 11. Distribution of á) protein and b) tritium after SDS-acrylamide gel electrophoresis of reduced AchE purified by chromatography on MAC-Sepharose 4B and labeled with ^{3}H-DIFP. AchE was denatured in the presence of 40 mM dithioerythritol. Gels were stained with Coomassie Blue, scanned for absorbance at 550 nm, sliced into sections and assayed for radioactivity. Molecular weights are averages from duplicate gels.

Figure 11a

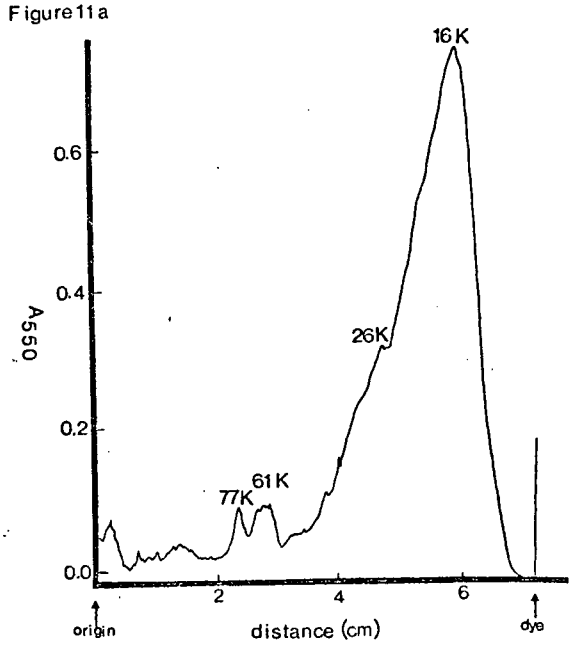
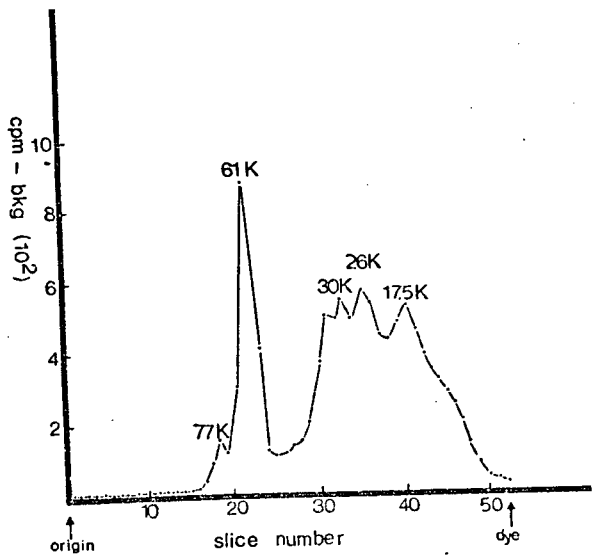


Figure 11b



d) Sedimentation in isokinetic gradients

The results of isokinetic sedimentation of AchE purified by chromatography on MAC-Sepharose 4B are shown in Figure 12. AchE activity appeared as one major peak with a corresponding value of 4.2 S (4.1-4.3S). The preparations labeled with ^{3}H-DIFP in the presence and in the absence of 125 μM-neostigmine each contained a single peak of radioactivity sedimenting at 5.6 S (5.3-5.9S) and 5.0 S (4.6-5.4S) respectively. These peaks all represent the same sedimenting particle within experimental error. It was concluded that neostigmine did not inhibit completely the binding of DIFP to AchE and that all of the radioactivity recovered from sedimentation of ^{3}H-preparations are located in AchE.

e) Isoelectric focusing

A gradient from pH 3-9.5 was obtained consistently after thin layer isoelectric focusing. Standard proteins focused at pIs reported previously (Radola, 1973). Figure 13 shows the result of a typical isoelectric focusing experiment. The isoelectric pH of AchE prepared by chromatography on MAC-Sepharose 4B was 5.3 ± 0.1. The single predominant protein band lacked AchE activity and focused at pH 9.2 ± 0.1; only a faint stain was detected at pH 5.3. An irreproducible peak of AchE activity focused at pH 6.7-7.7 (usually at pH 7.0). The peak appeared in most preparations but rarely focused at the same pH. Preparative scale experiments, in which 400-500 units of enzyme activity were applied to a 2-10 mm thick dextran layer, resulted in yields of less than 20 units after recovery of the enzyme by elution of the dextran

Figure 12. Elution profiles obtained after isokinetic sedimentation of standard proteins and AchE. Samples contained: a) 54 units of AchE activity (195 units mg^{-1} protein) purified by MAC-Sepharose 4B, b) the same preparation as a) but quantitatively inhibited by 10^{-4} M-^{3}H-DIFP, and c) the same preparation as a) but treated with 10^{-4} M-^{3}H-DIFP in the presence of 125 μM-neostigmine. Fractions (0.5 ml) were assayed for AchE activity (a) or radioactivity (b and c) (▲——▲), and β-galactosidase (S=15.9) activity (o——o); absorbance at 405 nm was measured to localize catalase (S=11.3) (■ —— ■) and myoglobin (S=2.0) (□ —— □). The profiles were duplicated to obtain average S values.

Figure 12 a

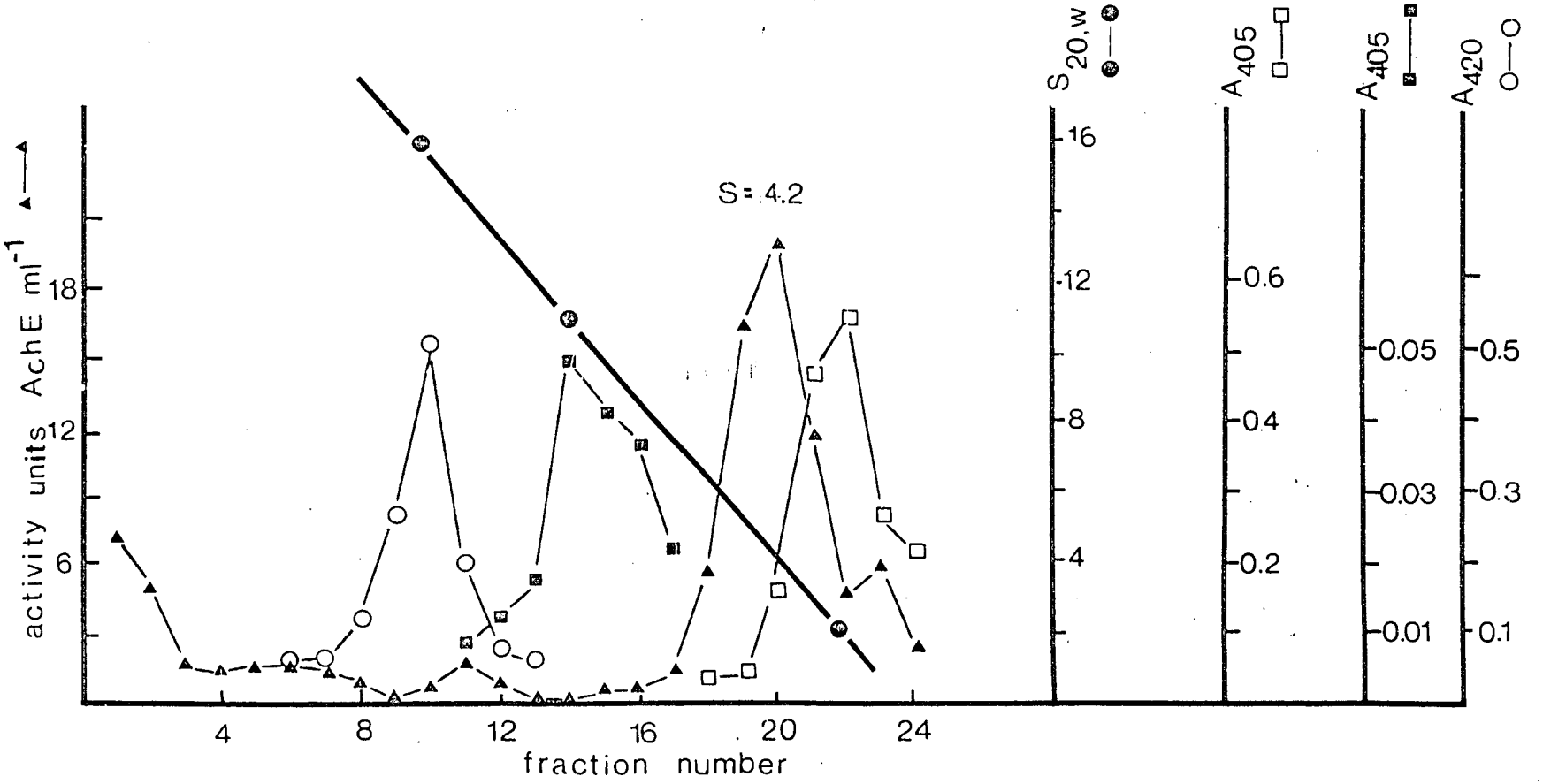

activity units AchE ml^{-1}
18
12
6
S = 4.2
4
8
12
16
20
24
fraction number
$S_{20,w}$
16
12
8
4
A_{405}
0.6
0.4
0.2
A_{405}
0.05
0.03
0.01
A_{420}
0.5
0.3
0.1

Figure 12b

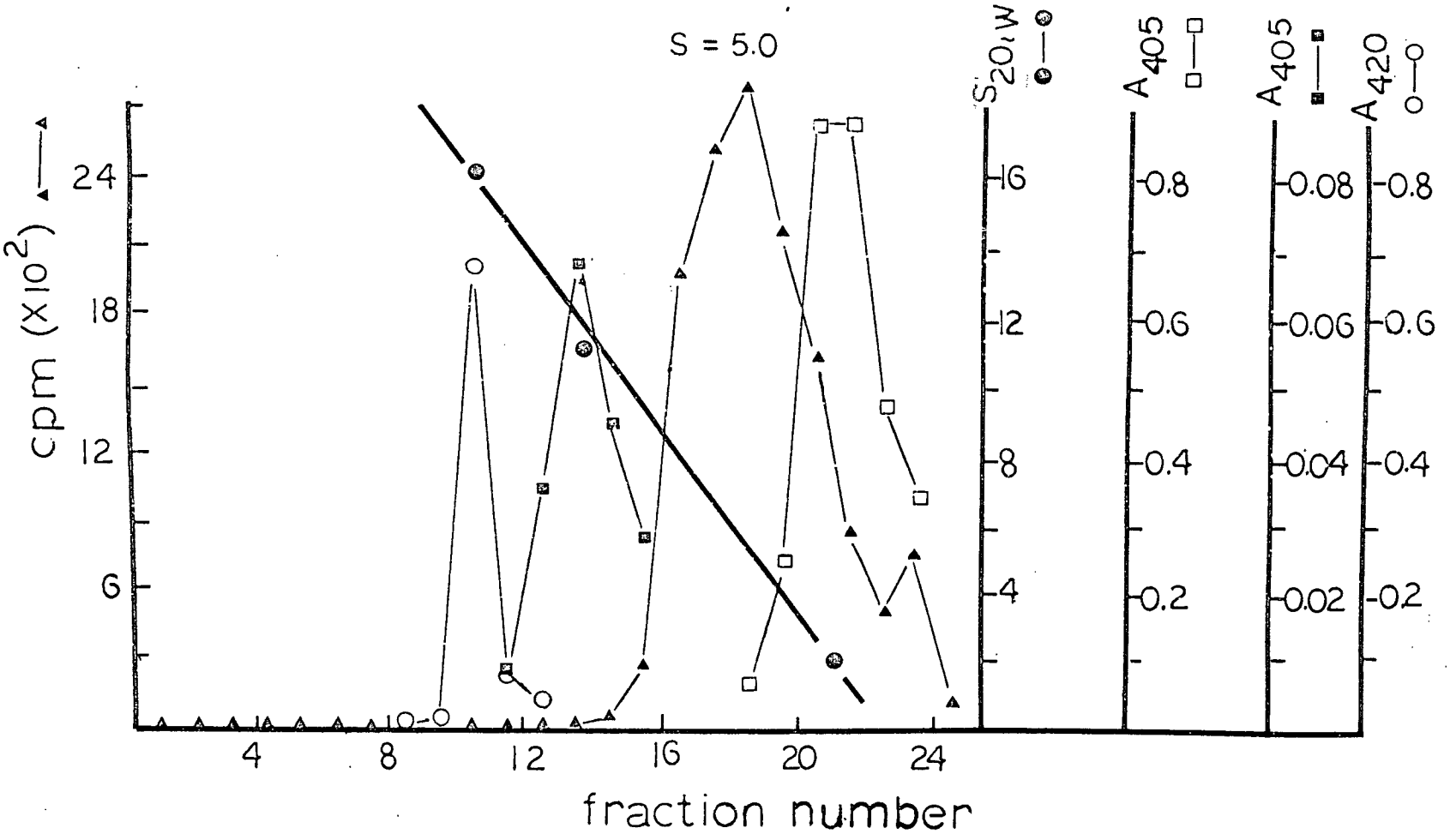
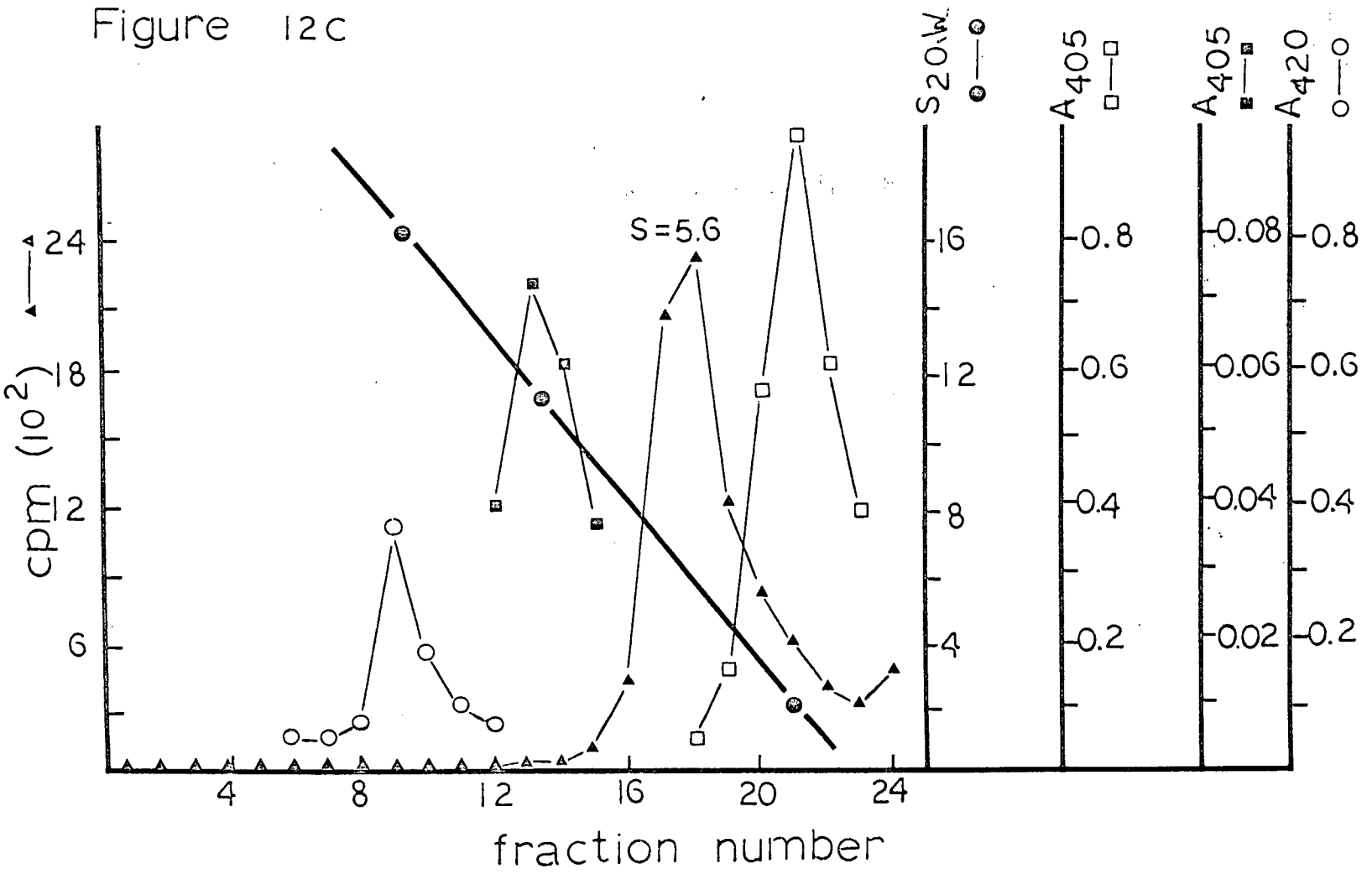

S = 5.0
cpm (X10²)
24
18
12
6
4
8
12
16
20
24
fraction number
S20,w
16
12
8
4
A405
0.8
0.6
0.4
0.2
A405
0.08
0.06
0.04
0.02
A420
0.8
0.6
0.4
0.2

Figure 12c


S=5.6
cpm (10^2)
24
18
12
6
$S_{20,w}$
16
12
8
4
A_{405}
0.8
0.6
0.4
0.2
A_{405}
0.08
0.06
0.04
0.02
A_{420}
0.8
0.6
0.4
0.2
4
8
12
16
20
24
fraction number

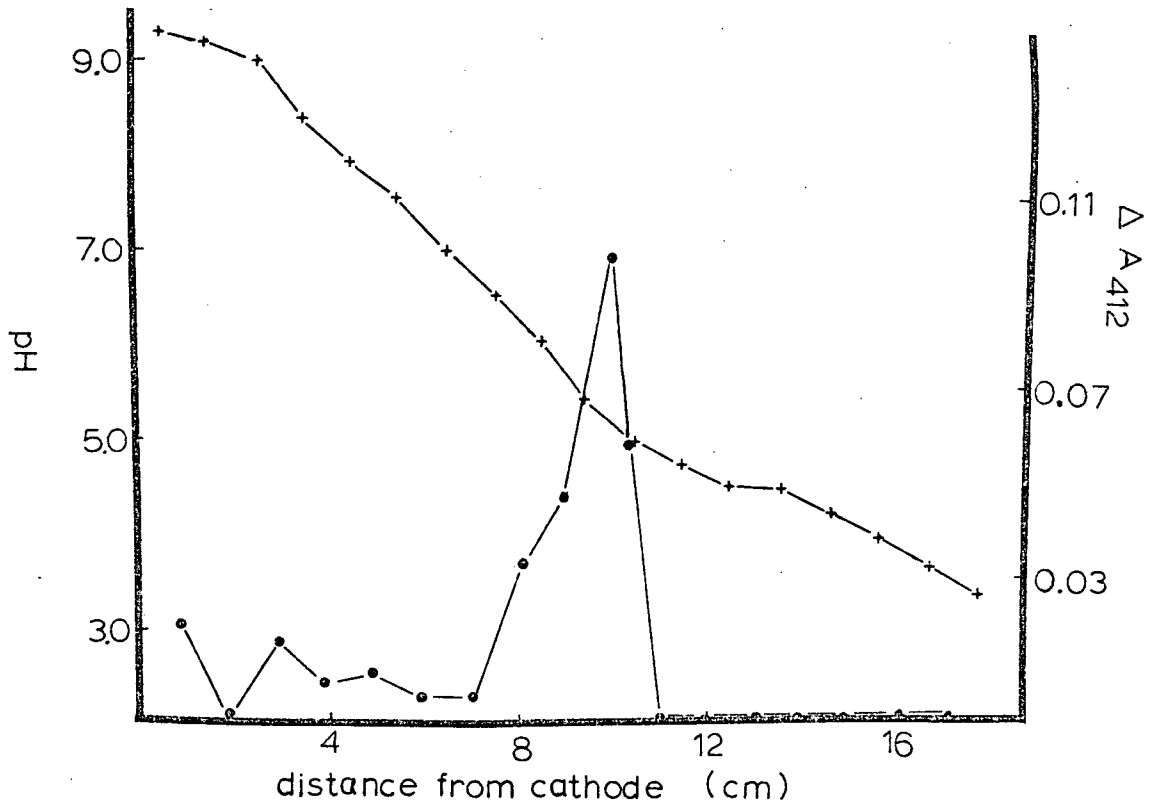


Figure 13. AchE activity and pH gradient after thin layer isoelectric focusing of enzyme purified by chromatography on MAC-Sepharose 4B. The 1 ml sample (480 units, 0 205 units mg^{-1} protein) was applied as a band 8 cm from the cathode and focused for 8 h at 200 V and an additional 10 h at 500 V. The pH gradient (+ —— +) and AchE activity (● ——○●) were determined on approximately 100 µl samples removed from the dextran layer and diluted with 200 µl of water.

on Sephadex G-25 or filtration through glass wool.

Figure 14 shows the results of isoelectric fosucing in a sucrose gradient. The peak of AchE activity at pH 7.0 accounted for less than 8% of the applied activity. Protein precipitate was observed in the column. Neither method of isoelectric focusing was suitable for further purification of AchE activity because of low recovery and precipitation.

f) Stokes Radius and Molecular Weight

The Stokes radius calibration curve obtained by gel filtration of standard proteins is shown in Figure 15. Using the value of K_{av} = 0.647 obtained for AchE prepared by method A, the Stokes radius of this enzyme was 4.00 nm. The calculated molecular weight of AchE was 76 000 ± 2000 using the value of S = 4.2 (Figure 12a) and assuming a $\bar{v}$ of 0.75 $cm^3 g^{-1}$ (Bon, et al., 1973). The frictional ratio, f/fo, resulting from these data was 1.37. A mol. wt. of 78 000 ± 8000 was obtained from a globular protein calibration curve of the same standard protein K_{av} data prepared by plotting log mol. wt. vs K_{av} (Figure 16).

g) Substrate Affinities and the Effect of Various Substances on AchE Activity.

1) The effect of acetylthiocholine, propionylthiocholine, and butylthiocholine on AchE activity.

The effects of acetylthiocholine, propionylthiocholine, and butylthiocholine on the AchE activity of MAC-Sepharose 4B-purified preparations are shown in Figure 17. The specific activity of AchE for hydrolysis of the three substrates (1mM) is shown in Table VI. Figure 18 shows a Lineweaver-Burke plot of the results shown in Figure 17 for acetylthiocholine. A regression line for points of the ascending

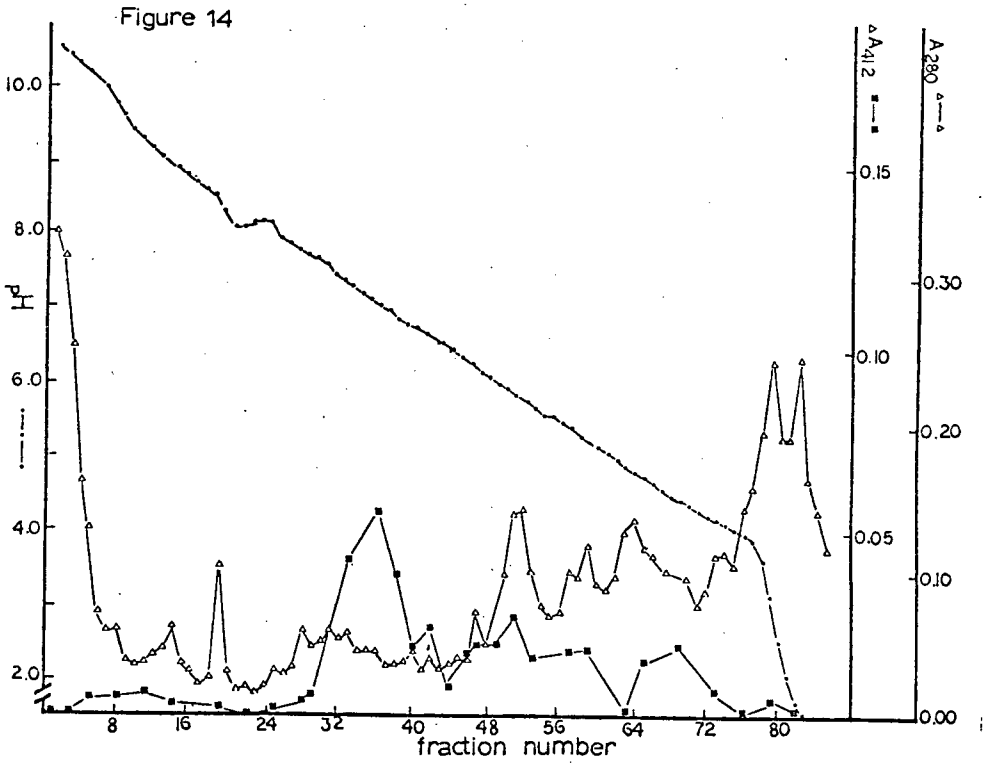


Figure 14. Elution profile after column isoelectric focusing of AchE purified by chromatography on MAC-Sepharose 4B. The sample was focused as described in Materials and Methods and the pH (● —— ●), the A_{280} (Δ ———— Δ), and the AchE activity (■ ——— ■) of each fraction were measured.

Figure 15. Stokes radius calibration curve. The plot was obtained after gel filtration of standard proteins on Sepharose 6B.

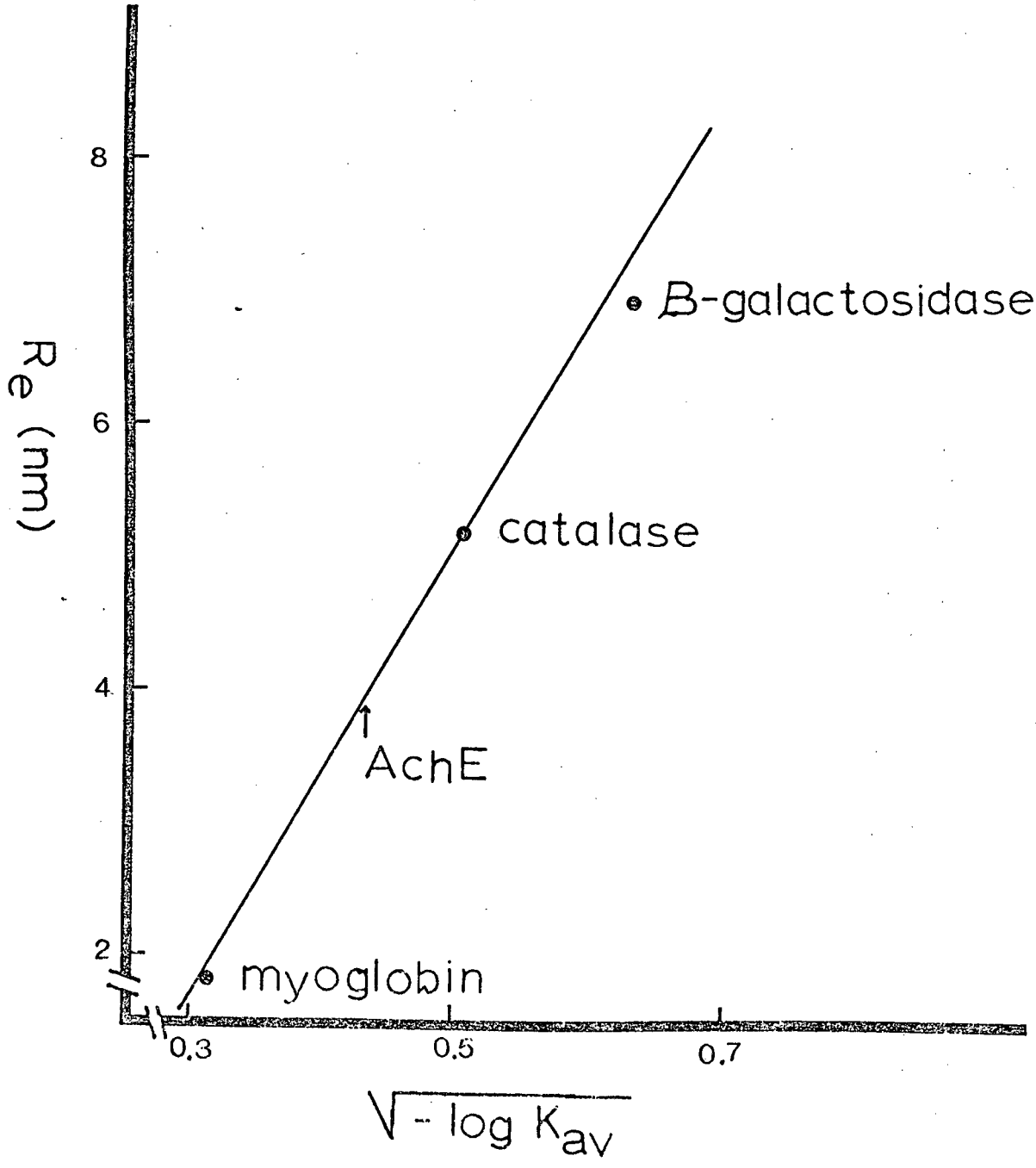


Figure 16. Globular protein molecular weight calibration curve. The plot was obtained after gel filtration of the standard proteins shown in Figure 15 on Sepharose 6B.

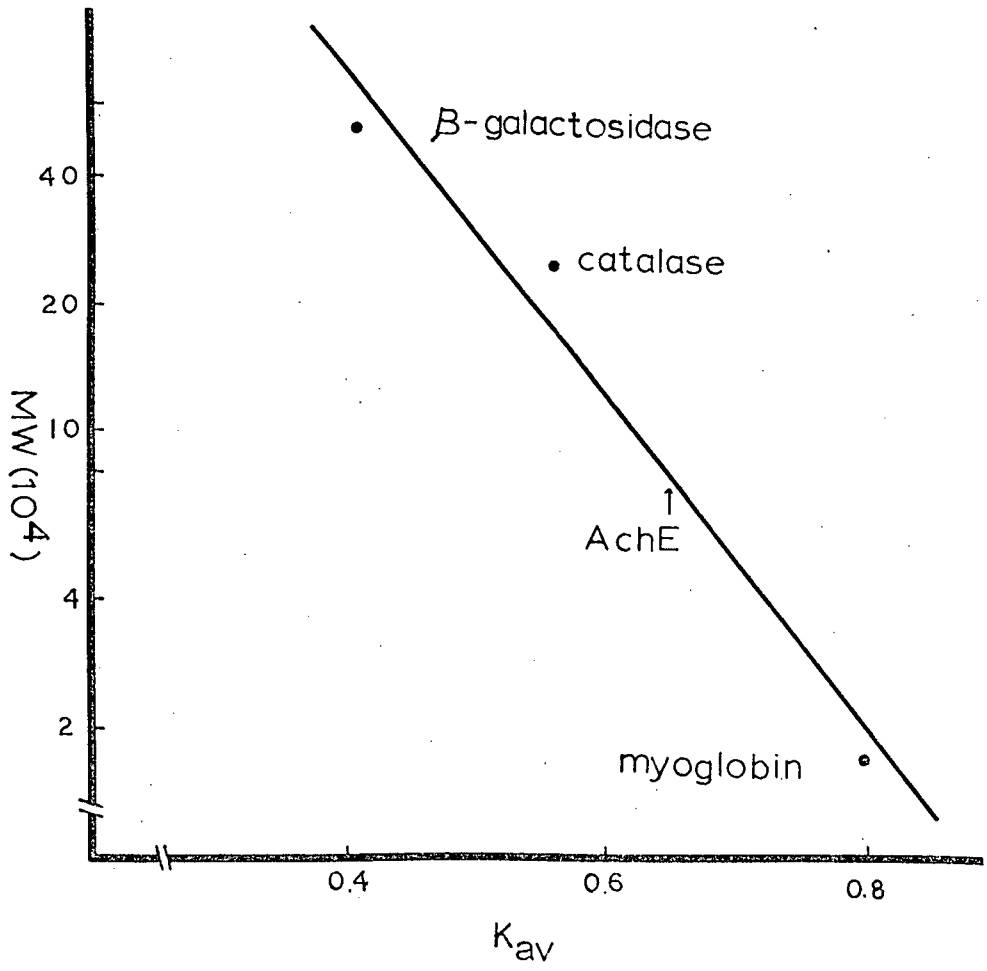


Figure 17. The effect of substrate concentration on AchE activity of MAC-Sepharose 4B-purified preparations. Values are averages of duplicate assays. Substrates tested included acetylthiocholine (+ —— +), propionylthiocholine (• —— •), and butylthiocholine (x —— x).

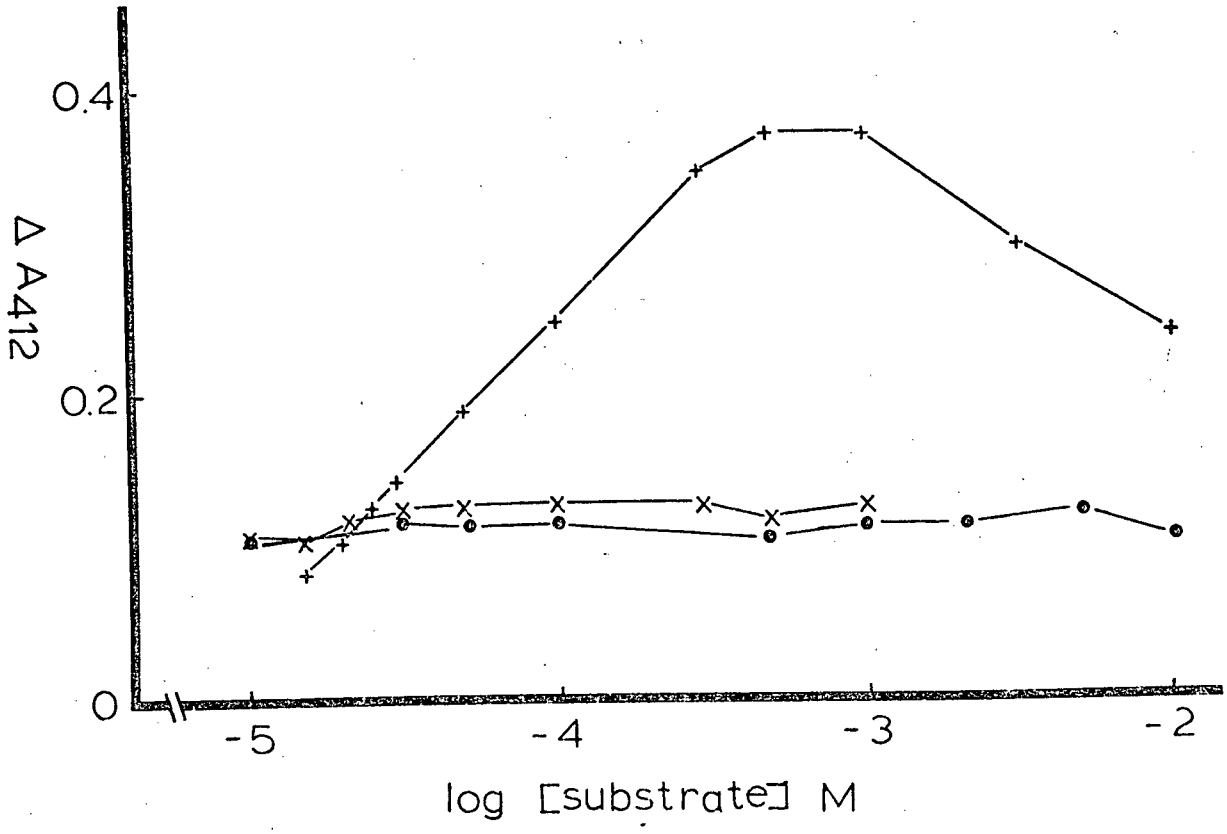
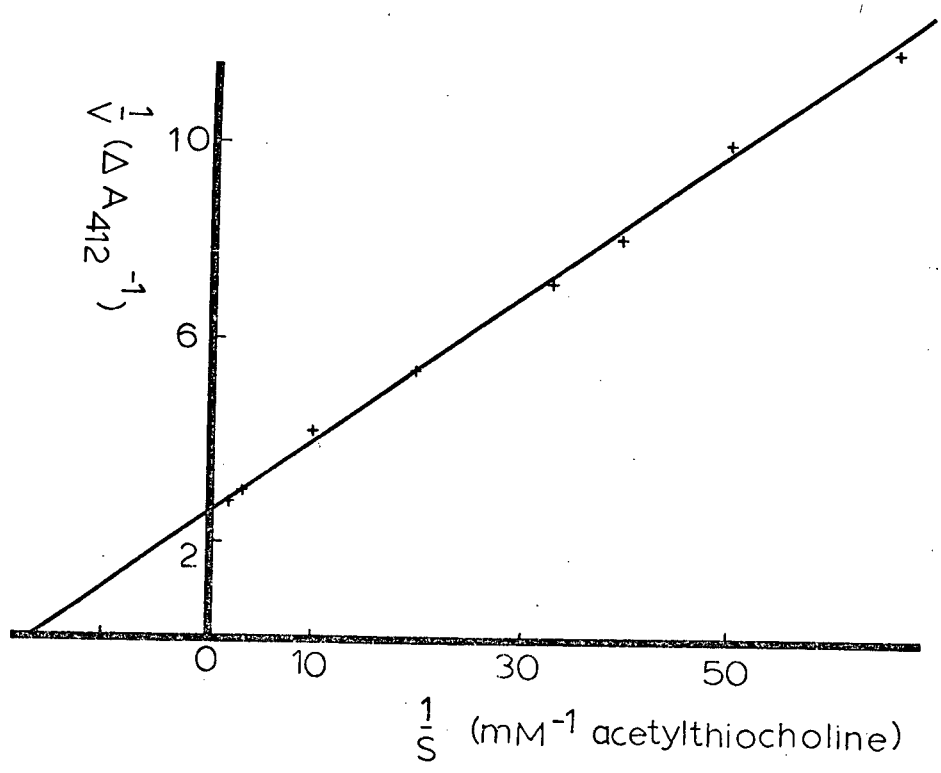


Table VI: Substrate specificity of AchE prepared by chromatography on MAC-Sepharose 4B for three choline esters. Values are means of duplicate assays.

Substrate	Specific activity (units mg^{-1} protein)
ACETYLTHIOCHOLINE	194.1
PROPIONYLTHIOCHOLINE	62.1
BUTYLTHIOCHOLINE	65.9

Figure 18. A Lineweaver-Burke plot for acetylthiocholine. Values are averages of duplicate assays, based on results presented in Figure 17.



limb of the acetylthiocholine curve in Figure 17 resulted in a K_m of 56 µM. The K_m for acetylthiocholine, determined by the same method on low specific activity (11.5 units mg protein^{-1}) preparations, was 58 µM. The enzyme did not obey Michaelis-Menten kinetics when either propionylthiocholine or butylthiocholine was used as the substrate. Substrate inhibition is characteristic of acetylcholinesterases from both plant and animal sources (Augustinsson and Nachmansohn, 1949; Wilson and Bergman, 1950; Riov and Jaffe, 1973; Kasturi and Vasantharajan, 1976) and both the high and low specific activity preparations from P. vulgaris roots displayed this characteristic (Figure 17).

2) The effect of choline, decamethonium, $(NH_4)_2SO_4$, and NaCl on AchE activity.

Figure 19 shows the effect of choline on AchE activity. Choline enhanced AchE activity at concentrations ranging from 0.5-10 mM. The enhancement was reduced at concentrations exceeding 10 mM.

Decamethonium has been used as an eluant in affinity chromatography studies involving the use of the N-methylacridinium ligand (Dudai, et al., 1972a). The inhibition of the AchE activity by decamethonium is shown in Figure 20. Activity was recovered following dialysis against three changes of buffer for 9 h at 4°C.

Figure 21 shows the effect of NaCl on AchE activity of preparations partially purified by method B. Both root and hypocotyl AchE activity were inhibited by high concentrations of NaCl. Inhibition could be reversed by dilution of NaCl. There was no interference with the AchE assay when the NaCl concentration was reduced to less than 0.1 M in the assay solution.

Figure 19. The effect of choline on MAC-Sepharose 4B-purified AchE (195 units mg^{-1} protein). The values are means of duplicate assays.

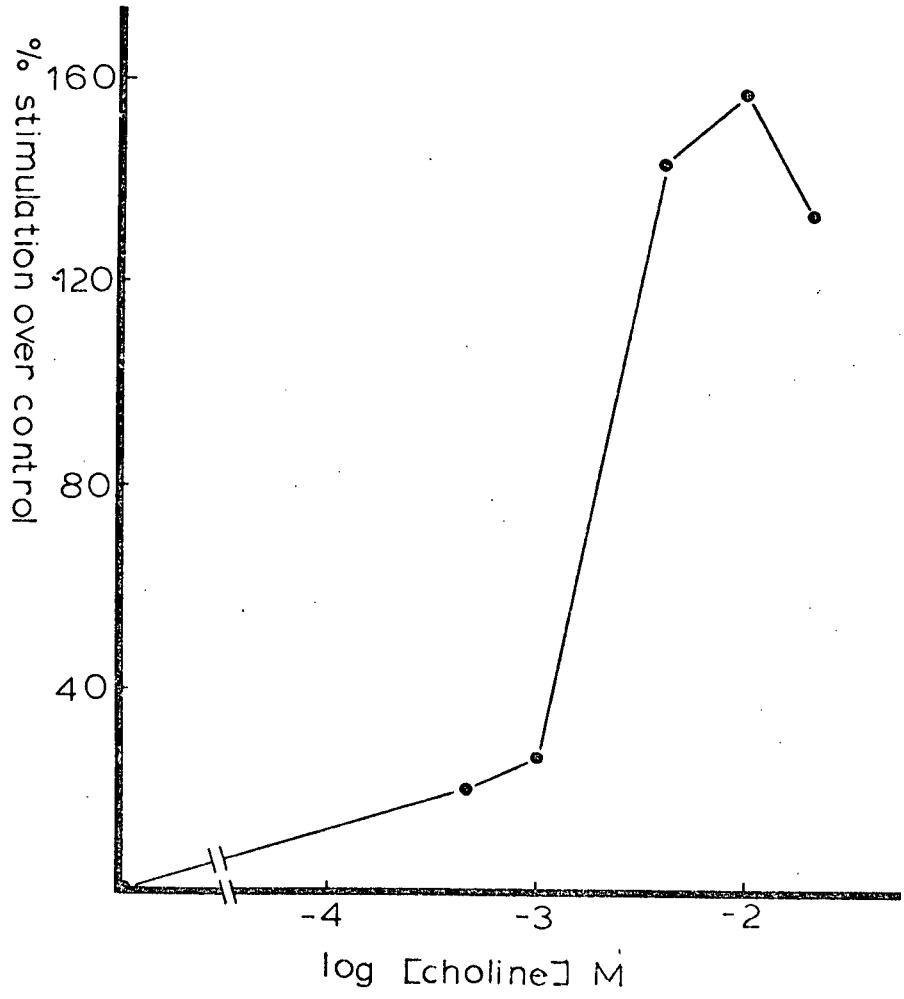


Figure 20. The effect of decamethonium on AchE activity. The values are means of duplicate assays of samples prepared by method A.

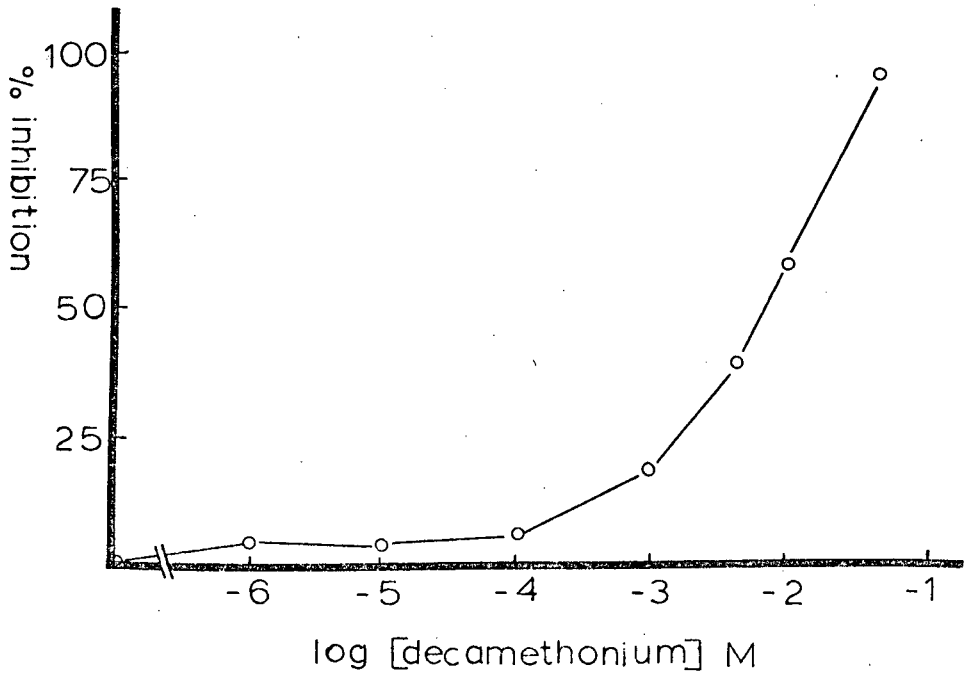
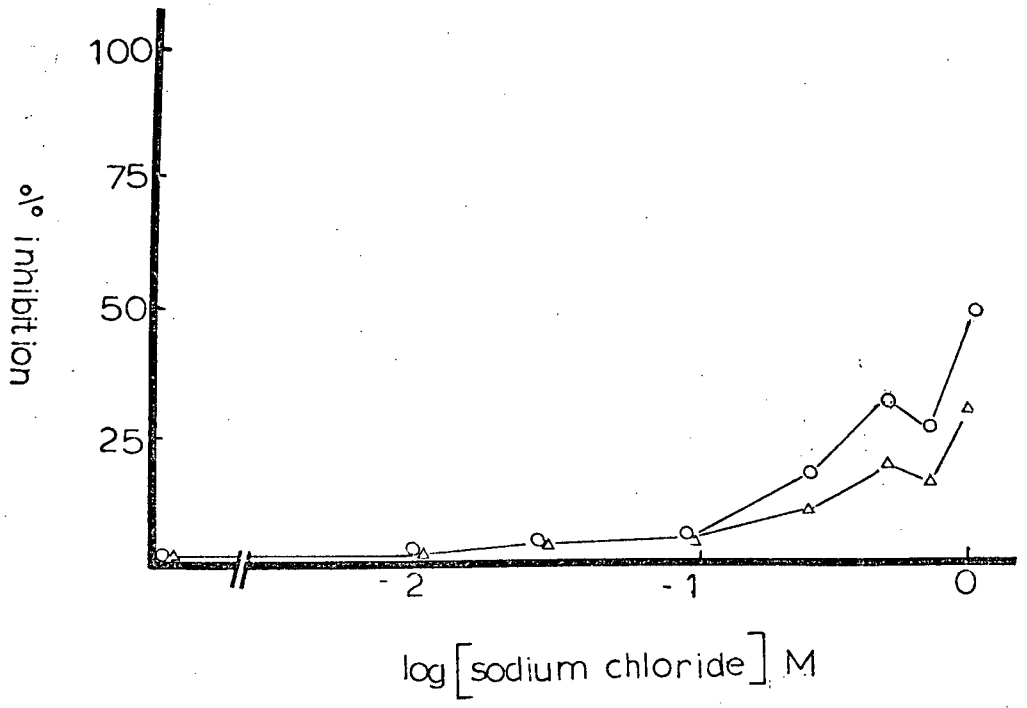


Figure 21. The effect of NaCl on AchE activity. Samples were prepared by 70% $(NH_4)_2SO_4$ precipitation of root (Δ —— Δ) and hypocotyl (o —— o) extracts. The values are means of duplicate assays.



High concentrations of $(NH_4)_2SO_4$ inhibited root AchE (Figure 22). This inhibition could be reversed by dilution of $(NH_4)_2SO_4$ but dialysis against buffer was preferred to regain activity.

Sodium azide (0.01% (w/v)) had no effect on AchE activity of preparations partially purified by method.

6. Behavior of Low Specific Activity AchE on Chromatographic Media

a) Chromatography on MAC-Sepharose 4B

The initial attempts to purify root AchE by affinity chromatography used conditions established for the purification of AchE from electroplaques of Electrophorus electricus (Dudai, et al., 1973; Webb and Clark, unpublished results). The results obtained are presented in Table VII. Little binding occurred when the enzyme was applied to the column in 10 mM-potassium phosphate buffer, pH 7.0, containing 1.0 M-NaCl. When the enzyme was applied in buffer without salt, 62% of the activity remained bound after a 20 ml buffer wash. All of that activity was recovered when the column was eluted with 1.0 M-NaCl in buffer. This step resulted in a 6.5 fold purification (Table VII). This purification factor suggested that under such conditions, the matrix was behaving as an ion exchanger. Conditions were modified in an attempt to obtain greater purification.

Samples prepared by method B in 10 mM-potassium phosphate buffer, pH 7.0, containing 0.5 M-NaCl were applied to 4 columns (1.5 X 1.0 cm) of MAC-Sepharose 4B containing 0.4, 1.0, 1.6 and 2.0 μmol of ligand ml^{-1}. The profiles shown in Figure 23 were obtained. Substantial activity was retarded only by the column containing 2.0 μmol of ligand ml^{-1}.

Figure 22. The effect of $(NH_4)_2SO_4$ on AchE activity. The values are means of duplicate assays of root extracts prepared by method B.

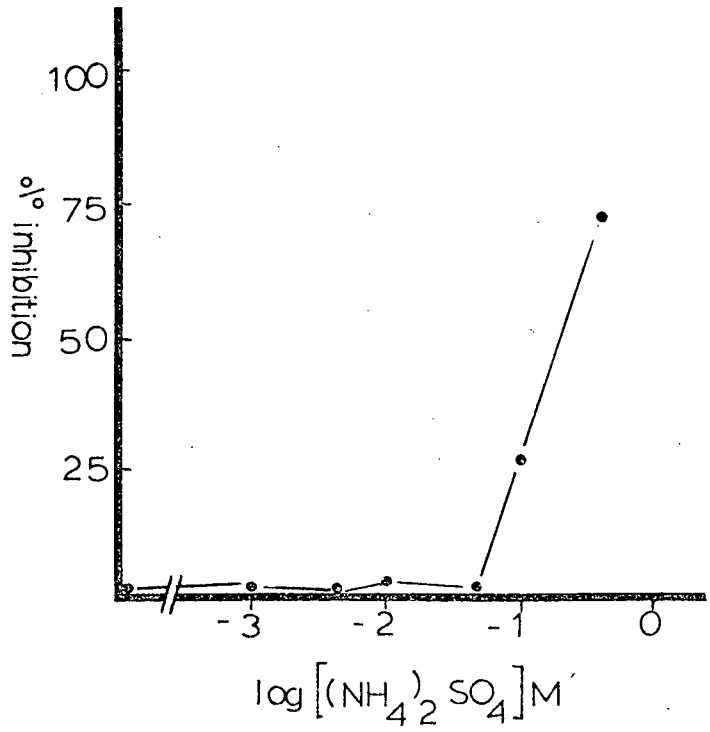
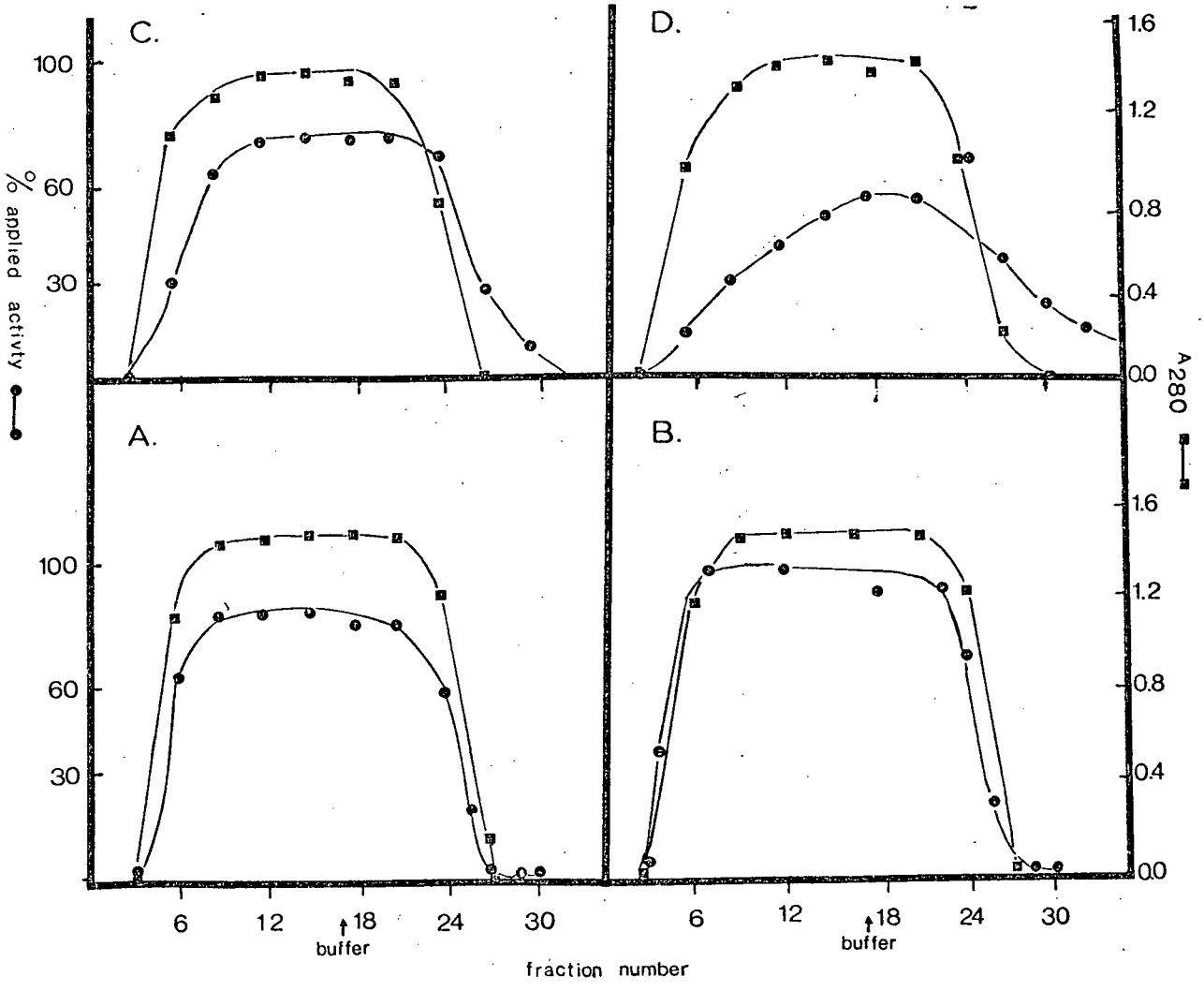


Table VII: Recovery of AchE activity from MAC-Sepharose 4B having a ligand concentration of 0.4 μmol ml^{-1}. Samples (8.8 units mg^{-1} protein) of root extracts prepared by method B were applied to a 1.5 ml column in 10 mM-potassium phosphate buffer, pH 7.0, either containing 1.0 M-NaCl or without NaCl. Values for recovery, specific activity and purification were obtained after elution of columns with buffer containing 1.0 M NaCl. Values are derived from averages of duplicate assays. N. D. denotes values not determined.

Equilibration Buffer	Activity Bound to column (% of applied activity)	Recovery (% of bound activity)	Specific Activity (units mg^{-1} protein)	Purification (-fold)
1 M-NaCl	6	N.D.	N.D.	N.D.
no NaCl	62	100	56.8	6.5

Figure 23. AchE activity recovered from four MAC-Sepharose 4B columns of differing ligand concentrations. Ligand concentrations in the columns were A) 0.4 μmol ml^{-1}, B) 1.0 μmol ml^{-1}, C) 1.6 μmol ml^{-1}, and D) 2.0 μmol ml^{-1}. Columns were loaded with 20 ml of enzyme prepared by method B in 10 mM-potassium phosphate buffer, pH 7.0, containing 0.5 M-NaCl. After all enzyme was applied, the columns were washed with 15 ml of equilibration buffer and eluate fractions (1 ml) were assayed for AchE activity (● —— ●) and A_{280} (■ —— ■).

Figure 23


C.
D.
A.
B.
100
60
30
% applied activity
1.6
1.2
0.8
0.4
0.0
A280
6
12
18
24
30
buffer
fraction number

Samples prepared by method B were dialyzed against 10 mM-potassium phosphate buffer, pH 7.0, containing either 0.05, 0.1, 0.2, or 0.4 M-NaCl. Each of these samples was applied to one of four columns containing 2.0 μmol of ligand ml^{-1}. The elution profiles are shown in Figure 24. The amount of protein bound to the columns varied inversely with the ionic strength of the buffering system. The greatest amount of AchE activity was bound to the columns operated in 0.2 M-NaCl. Elution of protein ceased abruptly after all 4 columns were washed with their equilibration buffers. Elution of AchE activity from the column operated in 0.4 M-NaCl continued after A_{280} values returned to baseline indicating that the enzyme was only retarded by the ligand. In the other three columns, enzyme activity was retained by the columns. No activity was recovered after the four columns were eluted with 5 mM-neostigmine and the resulting fractions were dialyzed against buffer for 36 h at 4°C. Following elution with neostigmine, the columns previously operated in 0.05 and 0.1 M-NaCl were eluted with buffer containing 1.0 M NaCl. No activity was recovered. It was concluded that neostigmine released the bound enzyme but the decarbamylation reaction was too slow to yield active enzyme after dialysis.

Enzyme prepared by method B was applied in 10 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl to columns containing 2.0 μmol of ligand ml^{-1}. The columns were eluted with either a NaCl gradient (0.2-1.0 M), 5 mM-acetylcholine, or a decamethonium gradient (0-50 mM). Fractions of the acetylcholine and decamethonium eluates were dialyzed against three changes of buffer for 10 h at 4°C prior to the AchE assay. The elution profiles obtained are shown in Figure 25a. Elution with the

Figure 24

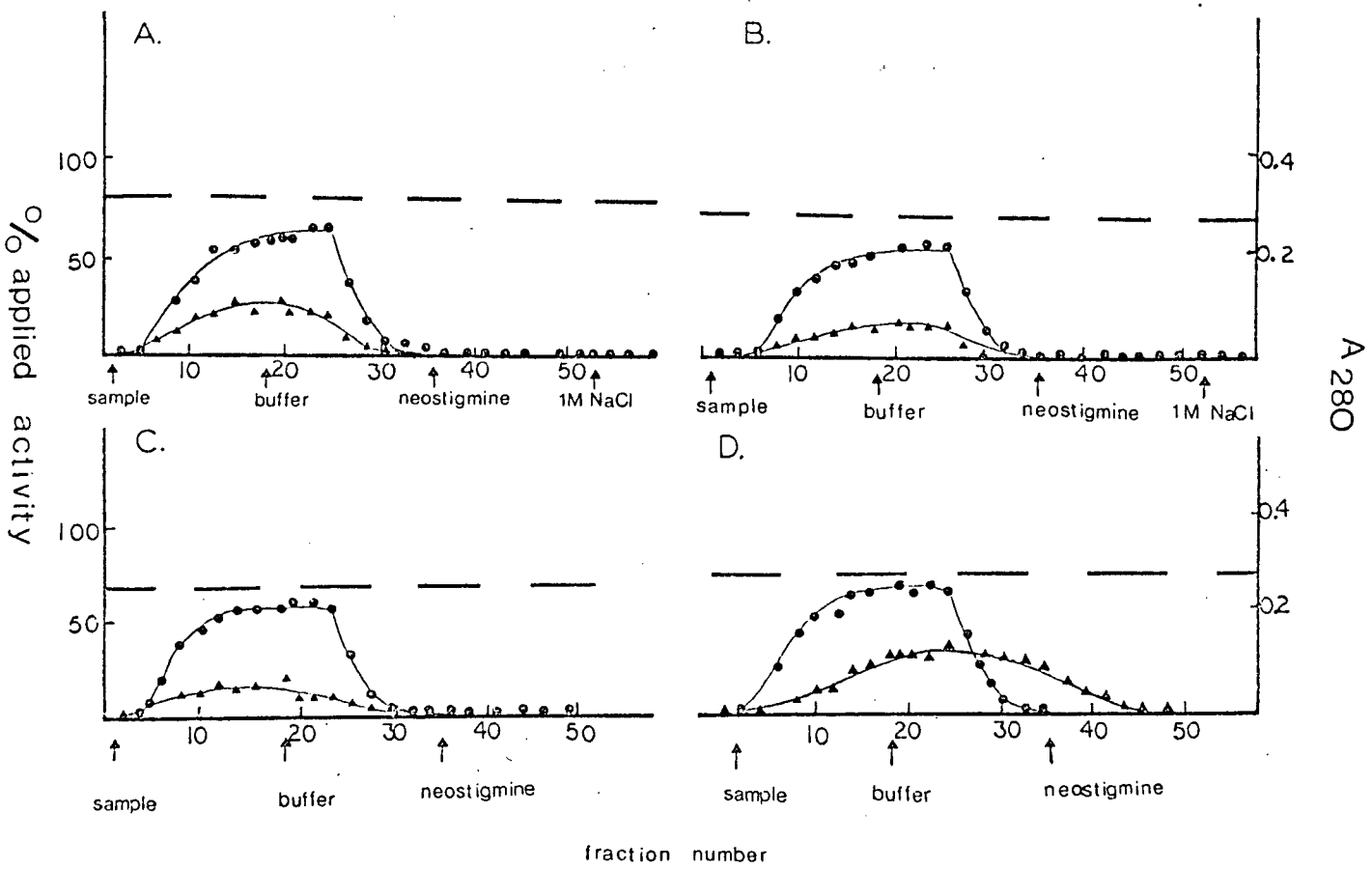


Figure 24. Recovery of AchE from MAC-Sepharose-4B columns equilibrated at different ionic strengths. Twenty ml of enzyme prepared by method B was applied to each of four columns in 10 mM-potassium phosphate buffer, pH 7.0, containing a) 0.05, b) 0.10, c) 0.20, or d) 0.40 M-NaCl. Each column was washed with its equilibration buffer and eluted with 5 mM-neostigmine in equilibration buffer. Columns a and b were then eluted with equilibration buffer containing 1.0 M-NaCl. Fractions (1 ml) were assayed for AchE activity (▲——▲) and A_{280} (● —— ●). The horizontal dashed lines indicate the A_{280}s of the applied samples.

Figure 25. Recovery of AchE from MAC-Sepharose 4B column by elution with a) a NaCl gradient (0.2-1.0 M), b) acetylcholine (5 mM) or c) a decamethonium gradient (0-50 mM). Thirty ml samples in 10 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl were applied to columns having a ligand concentration of 2.0 μmol ml^{-1}. Columns were washed, eluted with the appropriate eluant, and columns b) and c) were eluted with a NaCl gradient. Fractions (2 ml) were assayed for AchE activity (● —— ●) and A_{280} (■ —— ■). Profiles presented were duplicated.

Figure 25 a

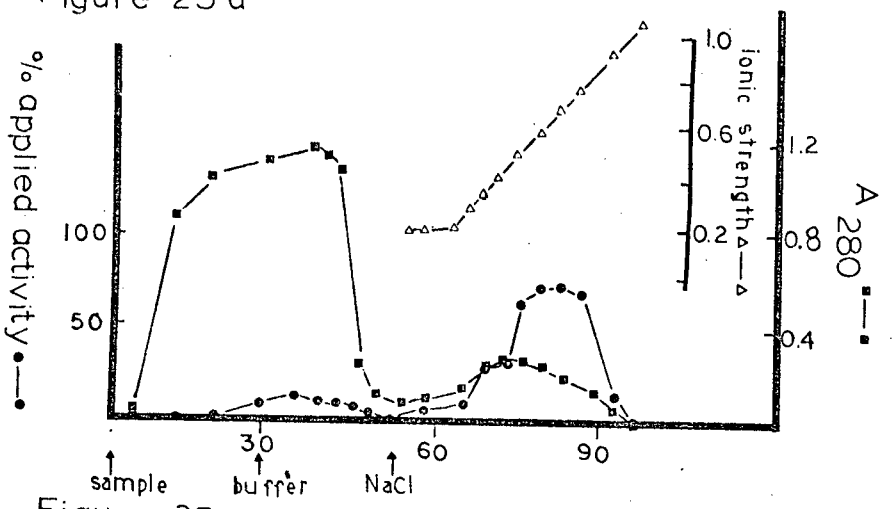


Figure 25 b

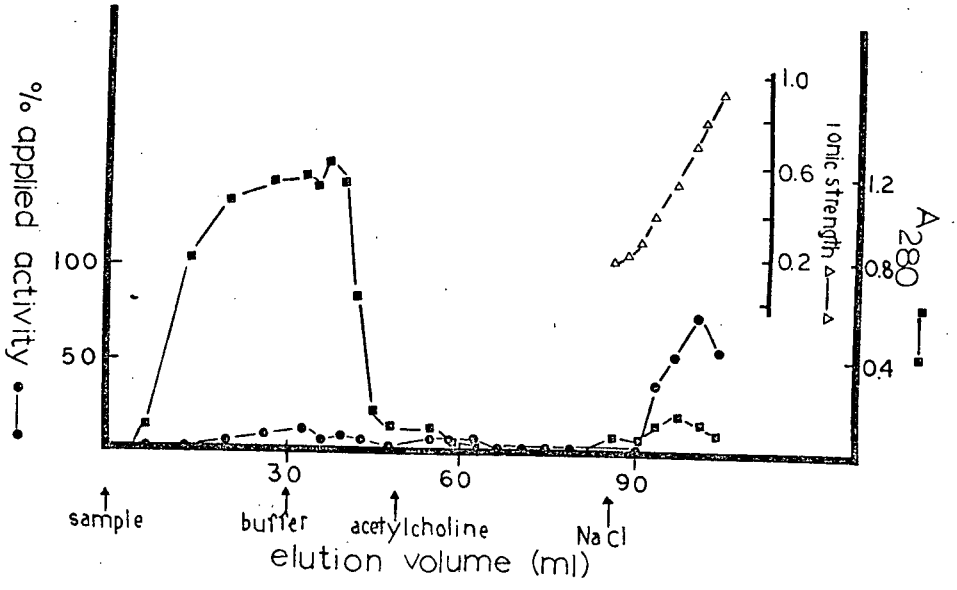
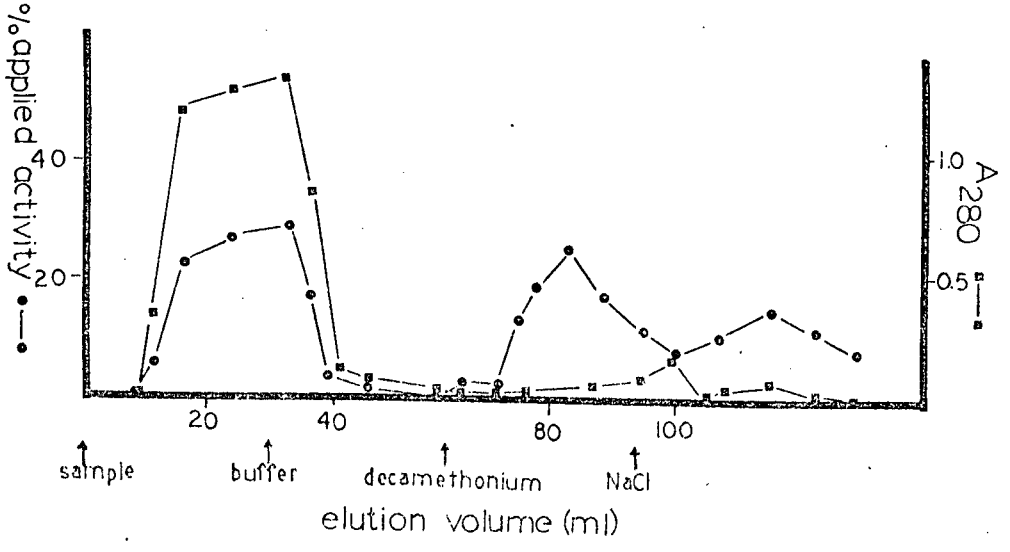


Figure 25 c



NaCl gradient produced a broad peak of relatively low specific activity (45 units mg^{-1} protein) accounting for 51% of the loaded activity. Similar results were obtained using enzyme prepared by method A.

No activity was recovered by substrate elution (Figure 25b). When a 0.2-1.0 M-NaCl gradient was subsequently applied, 68% of the bound activity was recovered. It was concluded that acetylcholine did not affect AchE binding.

Elution with a decamethonium gradient resulted in a slow emergence of protein and a peak of AchE activity at 25 mM-decamethonium (Figure 25c). This peak was asymmetric and broad suggesting non-specific elution. Twenty one percent of the bound activity was recovered in this peak and subsequent elution with the NaCl gradient yielded additional activity accounting for a total of 39% recovery. Specific activity was 60 units mg^{-1} protein. Comparable results were obtained from decamethonium eluates that had not been dialyzed prior to assaying. There was no additional recovery of AchE when 40 mM-decamethonium was applied after elution of all columns with 1.0 M-NaCl during routine purification (Figure 6).

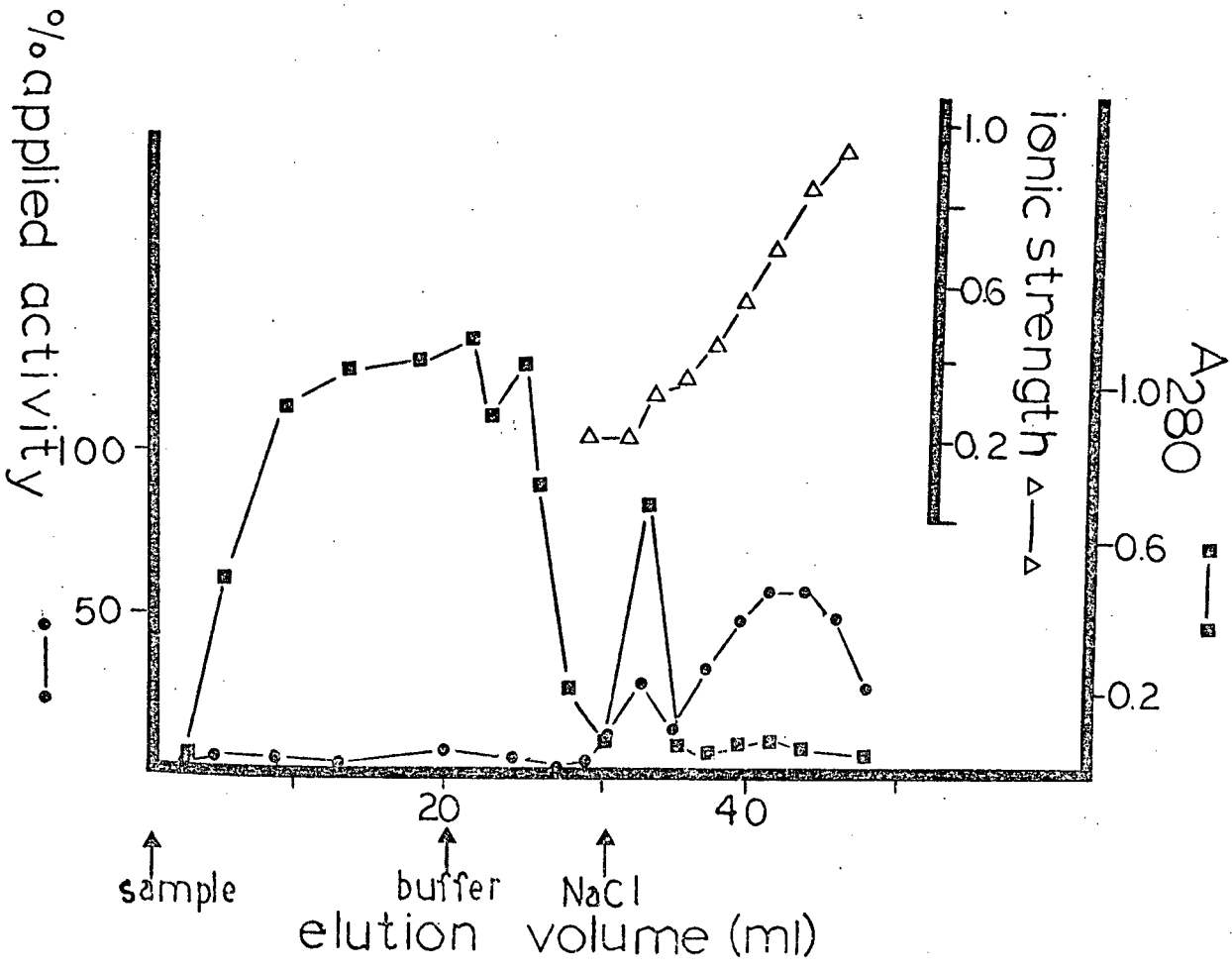
In the purification of eel AchE, an inverse relationship was found between the bed volume and recovery (G. Webb, personal communication). To examine this relationship in the plant enzyme, a comparison was made between two columns — 0.5 and 2.0 ml of MAC-Sepharose 4B (Table VIII). Greater recovery was obtained from the 2.0 ml column.

In one experiment (Figure 26) a delay of one day was allowed before application of the NaCl gradient. Most of the protein and a minor amount of AchE activity was released in the initial fraction. The major

Table VIII: Recovery of AchE activity in successive buffer (10 mM-potassium phosphate with 0.2 M-NaCl), decamethonium (0-50 mM) and NaCl, (0.2-1.0 M) gradient eluates as a function of bed volume of MAC-Sepharose 4B. Eluate activities are expressed as percentages of the applied sample volume. Values represent results from one of two duplicate experiments.

Bed volume (ml)	Buffer (%)	Decamethonium (%)	NaCl (%)	Total Recovery (%)
0.5	24	7	9	40
2.0	27	21	12	60

Figure 26. Recovery of AchE activity following delayed NaCl elution on a MAC-Sepharose 4B column. The column was developed as described for Figure 25 except one day passed between the completion of the buffer wash and the application of the NaCl gradient. AchE activity (● ——●) and A_{280} (■ ——■) were measured.



AchE peak contained a greater specific activity (67 units mg^{-1} protein) than the corresponding peak in Figure 25a.

b) Gel filtration on Sepharose 6B

Four major peaks of AchE activity were eluted when samples from both roots and hypocotyls prepared by method B were applied to a 25 X 50 cm Sepharose 6B column (Figure 27). No increase in specific activity was achieved. The first peak eluted in the void volume; the others had the following values for K_{av}: 0.48, 0.66 and 0.87. These value corresponded to mol. wts. of 1,000,000, 350,000, 70,000, and 10 000 daltons, respectively, applying the calibration curve of Figure 16, assuming that all species were globular.

c) Ion exchange chromatography on DEAE-Sepharose CL-GB.

Four major peaks of AchE activity were eluted from a DEAE-Sepharose CL-6B column (Figure 28). No substantial increase in specific activity occurred in any of these peaks. The elution profile suggests that there may be either isoenzymes or aggregates of AchE having different ion exchange properties.

There was no activity recovered from DEAE-Sepharose CL-6B when the column was operated at pH 7.2 and eluted with 1 mM-guanidinium or 1 mM-acetylcholine. The column was operated at pH 7.2 because any AchE having a pI of 7.0 would be loosely bound to the ion exchange resin thereby facilitating elution with the substrate (Scopes, 1977). The technique was not used to attempt to purify AchE having a pI of 5.3 because enzyme prepared by both methods (A and B) precipitated at pH 5.6 resulting in a loss of more than 90% of the enzyme activity.

Figure 27. Elution of AchE prepared by method B from Sepharose 6B. Fractions (5 ml) were collected and assayed for AchE activity (▲——▲) and A_{280} (□——□). The profile presented was reproduced in a separate preparation.

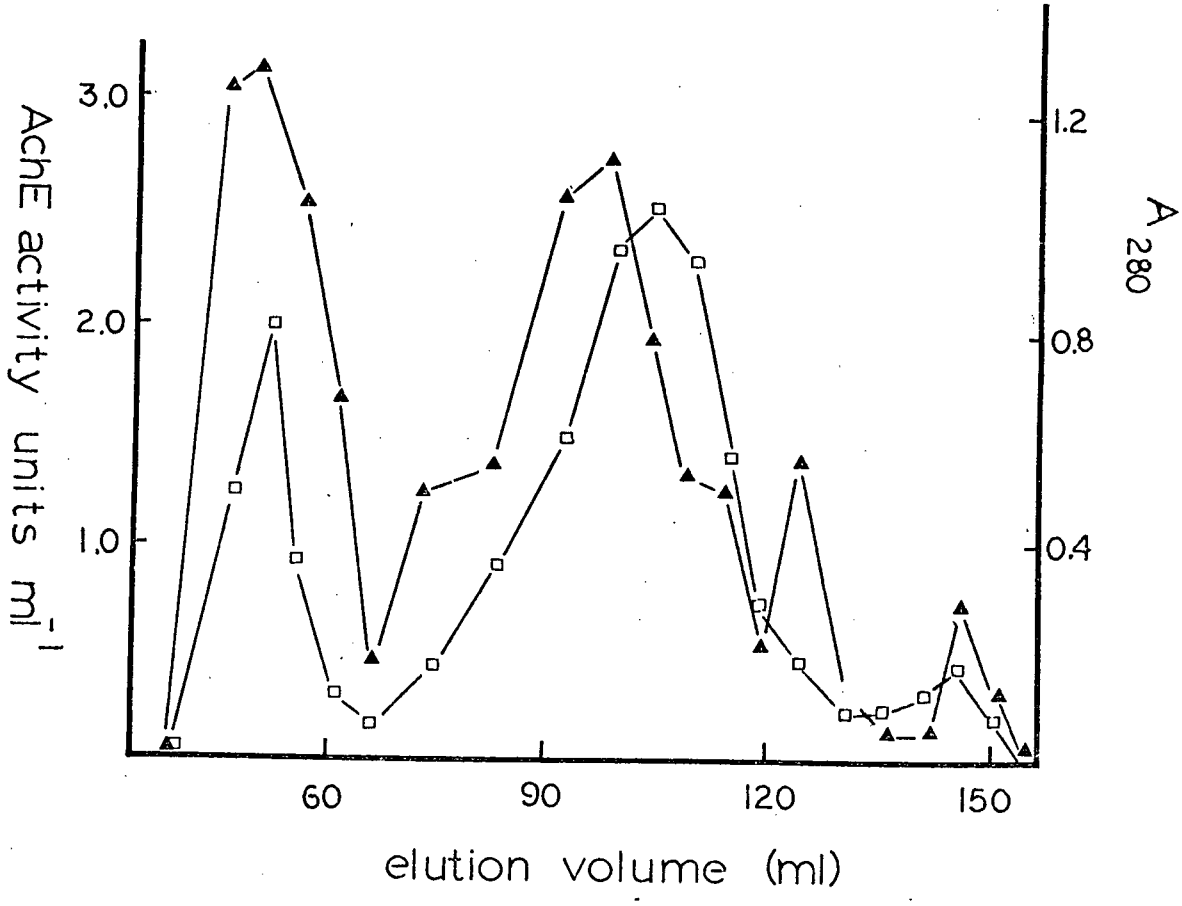
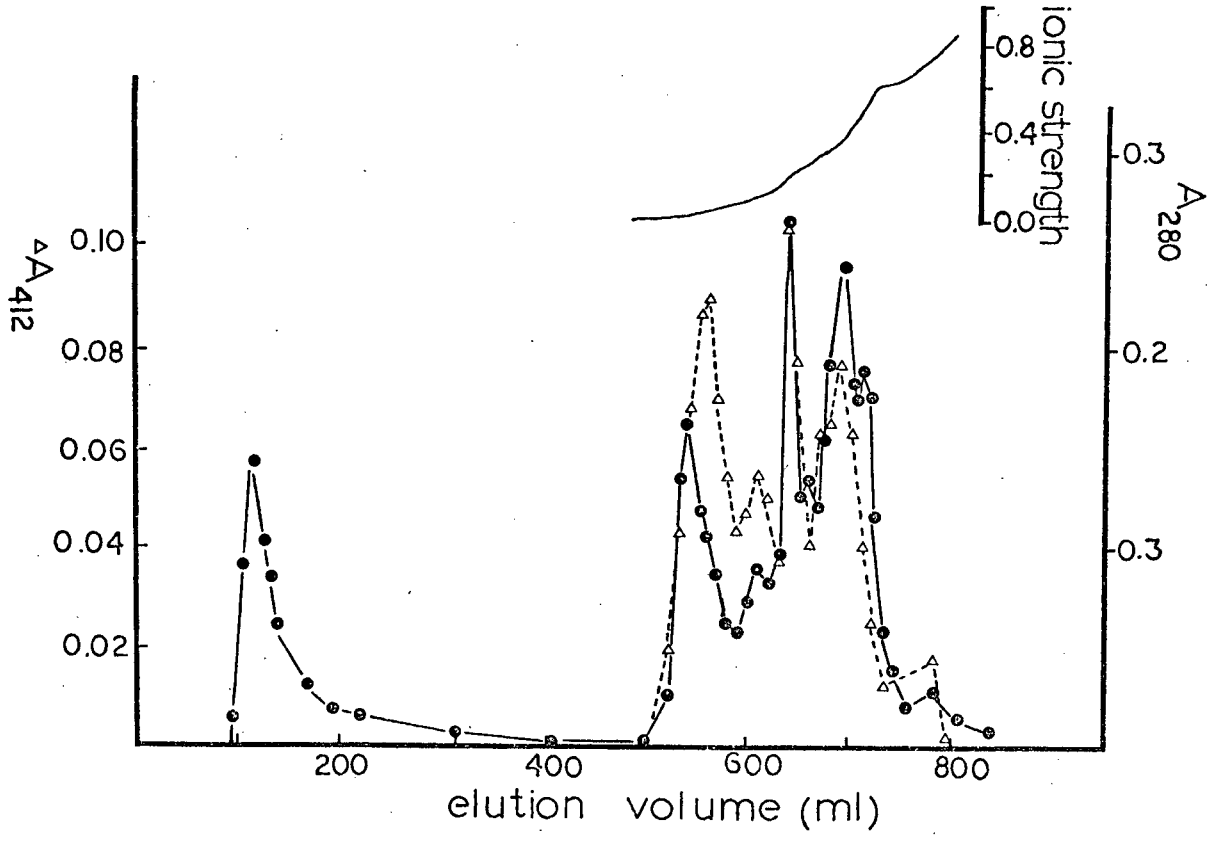


Figure 28. Elution profile of AchE eluted from DEAE-Sepharose CL-6B. AchE (540 units, 8.8 units mg^{-1} protein) was applied to the column in 20 mM-potassium phosphate buffer, pH 7.0, containing 0.03 M NaCl. After the addition of 315 ml of buffer, an NaCl gradient 0.03 to 1.0 M was applied. Five ml fractions were collected and assayed for AchE activity (Δ —— Δ) and A_{280} (● —— ●).



7. Physiological Role of AchE in the Hypocotyl

a) Effect of Growth Regulators on AchE Activity in the Hypocotyl Hooks

Experiments were designed to screen regulatory substances and white light for their effects on AchE activity in the hypocotyl hook. There was no effect of white light (1×10^3 erg $cm^{-2}sec^{-1}$) or Ethrel (100 ppm; 2-chlorosulphonic acid) on AchE activity or specific activity in hypocotyl hooks of 5-day old etiolated seedlings over a 4 day period (ie. 9 days after germination) (Figure 29). The Ethrel treated plants displayed the characteristic short hypocotyl and thick subapical region after one day. Kinetin (10^{-4} M) had no effect until the fourth day at which time a significant ($t_{4\ df} = 44.5$, $\alpha = 0.01$) decrease in specific activity of AchE was observed (Figure 30). Gibberellin (10^{-3} M) treated plants showed a significant ($t_{4\ df} = 28.4$, $\alpha = 0.01$) increase in AchE specific activity by the third day. By the fourth day plumular hooks of these and Ethrel-treated plants showed effects of tissue damage. The possibility that effects were an artifact of early stages of hook injury in Ethrel- and gibberellin-treated plants cannot be dismissed.

b) The Effect of Acetylcholine on the Hypocotyl

The effect of acetylcholine on the hook opening response of excised hypocotyl hooks was tested. The hook angle of the control hooks was $-46.5 \pm 2.9^{\circ}$ after 20 h in darkness (Figure 31). This value does not agree with the control value of 0° reported for the Black Valentine variety of bean typically used in hook angle experiments (Klein, 1956); however, no significant difference ($\alpha = 0.01$) was observed between the angle of hooks incubated in 10^{-3}, 10^{-5}, 10^{-7}, or 10^{-9} M-acetylcholine and the control hook angle. Hooks exposed to red

Figure 29. The effects of light and Ethrel on specific activity of AchE in hypocotyl hooks of 5-day old etiolated P. vulgaris. Plants were sprayed daily in dim green light with either water (▲——▲) or Ethrel (100 ppm; 2-chlorosuphonic acid) (■——■) and left in darkness or with water and exposed to continuous light (2 X 10^3 erg cm^{-2} sec^{-1}) (●——●). Hypocotyl hooks (15 to 20) were excised daily until 9 days after germination, homogenized, and assayed for AchE activity by the particulate assay procedure. Values are means of 3 experiments and error bars represent + or - S.E.M.

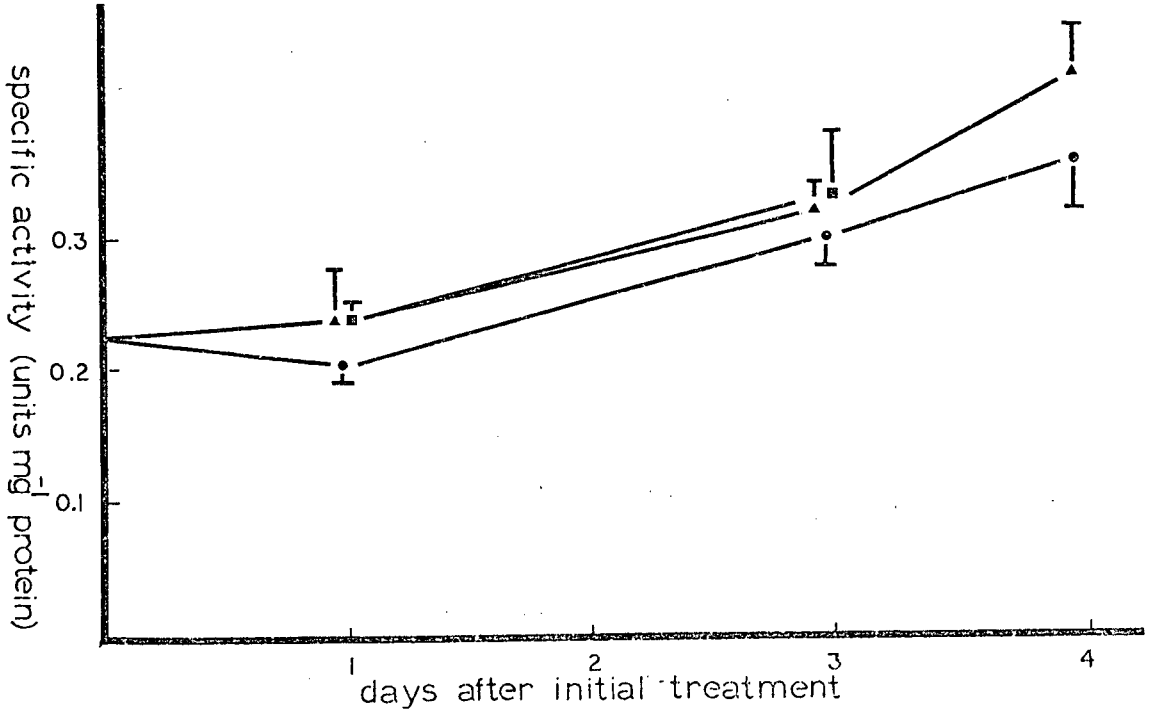


Figure 30. The effects of gibberellin and kinetin on specific activity of AchE in hypocotyl hooks of 5-day old etiolated P. vulgaris. Plants were sprayed daily in dim green light with either water (▲——▲), 10^{-3}M-gibberellin (●——●), or 10^{-4}M-kinetin (■——■) and left in darkness. Hypocotyl hooks were excised daily until 9 days after germination, homogenized and assayed for AchE activity. Values are means of 3 experiments and error bars represent + or - S.E.M.

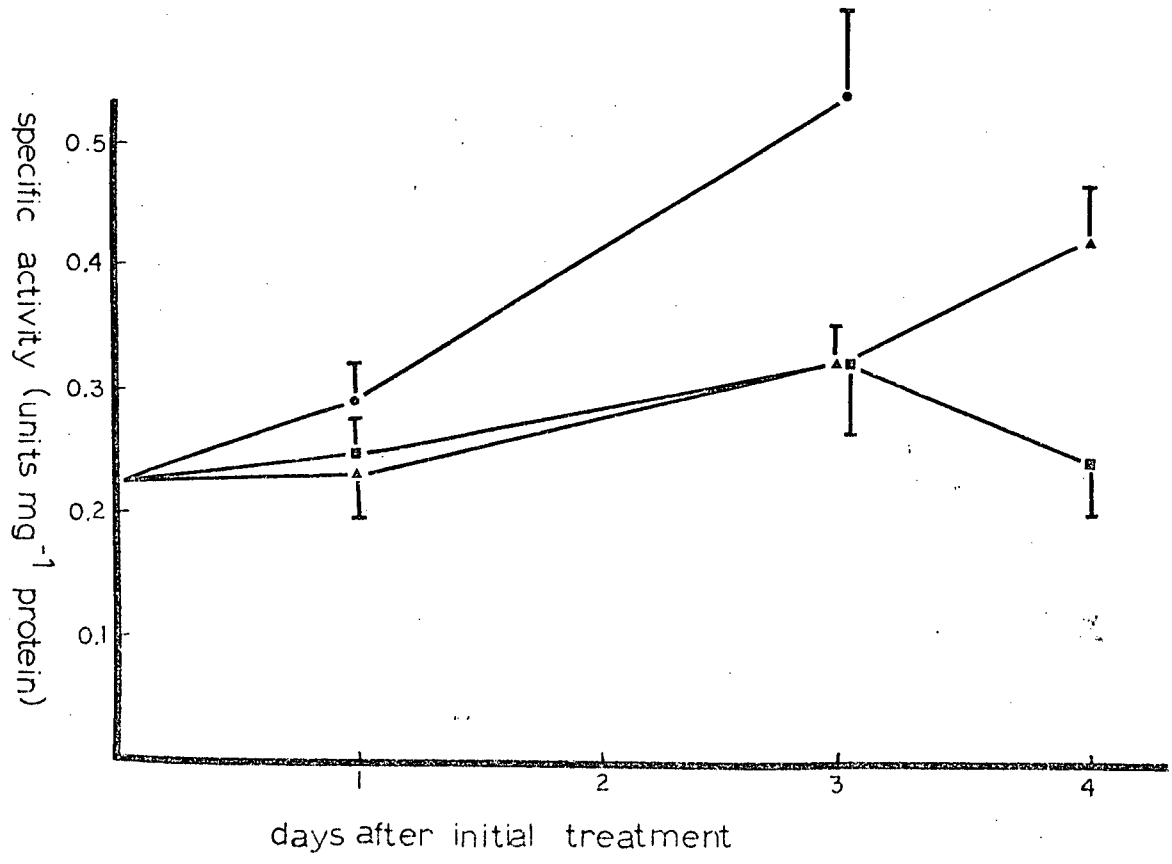
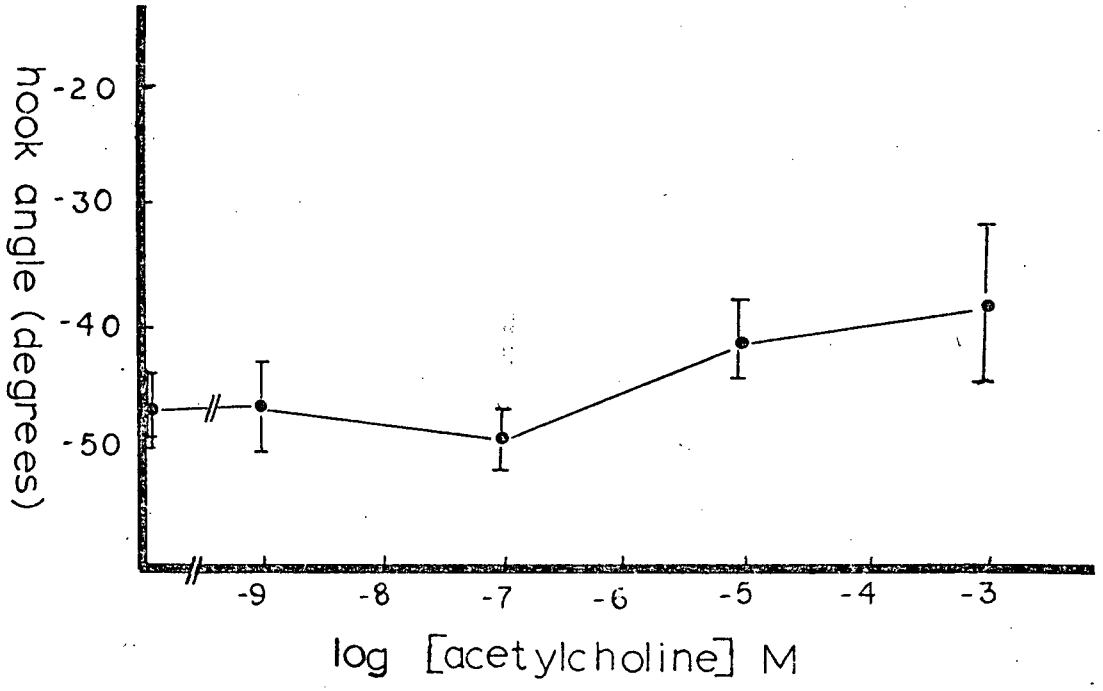


Figure 31. The effect of acetylcholine on the hook angle of 20 h excised hypocotyl hooks of P. vulgaris. Values are means of 30 individual hooks for two pooled experiments and error bars represent ± S.E.M.



light had a final angle of 72.4 ± 19.8°.

Seven-day old etiolated P. vulgaris hypocotyls elongated to 130.7 ± 0.8% and 130 ± 1.3% of their initial lengths after 24 h in darkness when sprayed with water or 10^{-6} M-acetylcholine, respectively. There was no significant difference ($\alpha = 0.01$) between these values.

D. DISCUSSION

1. Identity of AchE in P. vulgaris.

Criteria for the identification of AchE activity in plants have been established (Fluck and Jaffe, 1974a). They include inhibition by neostigmine and maximal activity against acetyl esters of choline as fundamental features of the enzyme. AchE activity that satisfied these criteria was identified in P. vulgaris.

Acetylcholine hydrolysis in the presence of partially purified extracts was inhibited by neostigmine (Figure 2) at concentrations effective against AchE activity in animals (Augustinsson, 1960 and 1963) and plants (Riov and Jaffe, 1973). This property was exploited in activity assays to correct for spontaneous or non-specific hydrolysis of acetylcholine and all of the esterase activity in preparations purified beyond $(NH_4)_2SO_4$ precipitation was neostigmine inhibitable. The neostigmine inhibitable hydrolysis of acetylthiocholine was related linearly to the volume of the extract which was assayed (Figure 4) and the assay time (Figure 3). The observation that acetylthiocholine was hydrolyzed three times as fast as either butyl- or propionyl-thiocholine (Figure 17) further supported the argument that this plant cholinesterase was an acetylcholinesterase.

Both acetylcholine and its thiocholine analogue used as the substrate in this study, have been reported to occupy the same position in substrate affinity and hydrolysis rate hierarchies when compared to other acylcholine esters. Both animal (Ellman, et al., 1961) and plant AchEs (Riov and Jaffe, 1973) hydrolyze the acetylthiocholine more slowly than acetylcholine and have slightly higher Kms for the analogue.

The possibility of microbial contamination of extracts as a source of AchE activity was considered because a cholinesterase was identified in the bacterium Pseudomonas fluorescens (Fitch, 1963; Laing, et al., 1967). The present study did not address this problem directly but the following observations negate this possibility. AchE activity was located histochemically within the cells of Phaseolus aureus roots (Fluck and Jaffe, 1974b) and was located in purified cell walls in this study. It was extracted in similar amounts from one experiment to the next, a feature not to be expected from contaminating elements. Callus tissue grown axenically produced activities comparable to those from the parent tissue. Surface sterilized seeds germinated in sterile petri plates produced seedlings having the same activity as their vermiculite-grown counterparts (R. A. Fluck, personal communication).

A group of cholinesterases exists in plants which has a low Km for acetylcholine or acetylthiocholine (56-200 μM) and is inhibited by the carbamates neostigmine (10^{-5}M) or eserine (10^{-2}M) (Tzagaloff, 1963; Riov and Jaffe, 1973; Fluck and Jaffe, 1974d; Kasturi and Vasantharajan, 1976). Variations exist in the response to choline and the hydrolysis rate hierarchy. The AchE in this study and the enzymes studied by Riov and Jaffe (1973) and Kasturi and Vasantharajan (1976) have been purified beyond $(NH_4)_2SO_4$ precipitation. Only the bean enzymes were

stimulated by choline (Figure 19 and Riov and Jaffe, 1973). All of the cholinesterases which have been partially purified by $(NH_4)_2SO_4$ precipitation are inhibited by choline (Tzagaloff, 1963; Schwartz, 1967; Fluck and Jaffe, 1975). Both Schwartz (1967) and Kasturi and Vasantharajan (1976) studied enzymes from Pisum sativum root but the latter workers did not examine the effect of choline on their preparations. Cholinesterase activity was identified in 23 species from 5 families (Fluck and Jaffe, 1974d). Which of these cholinesterases are acetylcholinesterase remains uncertain because of low level of purity of these preparations and the undetermined substrate hydrolysis rate hierarchy of each enzyme.

AchE was located in the roots of P. vulgaris and tentatively identified in the hypocotyl. The hypocotyl enzyme was not purified beyond $(NH_4)_2SO_4$ precipitation and must be considered as a cholinesterase even though it resembled the root enzyme in several respects (Figures 4, 21, and 27, and Table II). AchE has been identified and partially purified from roots of two other members of the Fabaceae (Riov and Jaffe, 1973; Kasturi and Vasantharajan, 1976).

2. Extraction and Localization of AchE

The highest extractable AchE activities have been reported in roots. Leaves contained nearly as much as roots; and buds, hypocotyls, and stems contained the least (Fluck and Jaffe, 1974b and d). Activity measured in P. vulgaris tissue under different conditions was always greater in the roots but quantitation of activity proved difficult. Enzyme activities were measured in situ by assaying tissue slices (Table III) but values obtained by this method were slightly (in

hypocotyls) or substantially (in roots) lower than activities in homogenates. This suggested that not all of the enzyme was accessible to the substrate and that the assay was not suitable for cholinesterase determinations in situ. Fresh homogenates contained substantially lower activities than the same homogenates after dialysis against water (Table I). The increase of activity during dialysis is attributed in part to the presence of an endogenous dialyzable inhibitor (Table I). However, other factors removed during dialysis contributed to the low activity since return of concentrated diffusate did not reduce activity to the level detected before dialysis. The inhibitory agent in the hypocotyl was greater than in the root. Insufficient information is available to determine the nature or the significance of its effect. Kasturi and Vasantharajan (1976) found increased total AchE activity after $(NH_4)_2SO_4$ precipitation. Fluck and Jaffe (1974d) identified a 2-nitro-5-thiobenzoate decolorizing activity in buffer extracts of P. aureus roots which was partially dialyzable and heat-inactivated. A similar decolorizing agent has been reported in sea urchin extracts (Wolfson, 1972) which was heat-inactivated and completely dialyzable. Regardless of the nature of the inhibitor values for AchE activity in fresh homogenate appear to be minimal. The presence of inhibitors of presumably variable quantity made assay of crude extracts difficult. Values were highly dependent on the extraction and assay procedures (see Tables I, II, and III; Figures 29 and 30) and no quantitative estimate was obtained for activities in situ.

Enzyme activity was detected in cell walls extracted from P. vulgaris hypocotyls. The result was expected since Fluck and Jaffe

(1974b) found that 95% of the AchE activity in P. aureus roots was located in the cell walls. Jansen, et al., (1960) showed that cell walls were capable of binding soluble proteins which were not native components of the wall. The AchE activity was considered a real component of the cell wall because the 10mM-buffer insoluble activity was greater than 5% of the total activity.

The 5% $(NH_4)_2SO_4$ extracted 43% of the root acetylcholinesterase (Fluck and Jaffe's (1974b) value was 37%) and 81% of the hypocotyl enzyme. The different efficacies of this extractant in roots and hypocotyls suggests that the localization or binding properties of AchE differed between these two organs. Ammonium sulphate also extracted a greater portion of AchE from aerial organs than from roots of light grown mung bean plants (Fluck and Jaffe, 1974b). The observation that only 57% of the light grown mung bean hypocotyl AchE (Table 6 in Fluck and Jaffe, 1974b), but 81% of the etiolated P. vulgaris hypocotyl enzyme was extraced further suggests that the developmental status of these plants affects the properties of AchE. These extraction data indicate that two populations of AchE (or cholinesterase) activity exist in the cell wall and they differ in their binding properties to the wall (Fluck and Jaffe, 1974b). At least five other cell wall enzymes exhibit this property and in the case of peroxidases, covalently and noncovalently bound populations of the enzymes have been proposed (Hall and Butt, 1968; Nevins, 1970; Ridge and Osborne, 1970; Klis, 1971; Copping and Street, 1972).

The selection of 5% $(NH_4)_2SO_4$ to extract AchE from P. vulgaris was based on work of Riov and Jaffe (1973), and Fluck and Jaffe (1974b)

who found 4% $(NH_4)_2SO_4$ to be the most effective of 10 extraction media. This was also the most effective extractant used in the purification of autolyzed or trypsin-digested eel AchE (Leuzinger and Baker, 1967).

3. Purification and Characterization

The use of 5% $(NH_4)_2SO_4$ favored extraction of one form of AchE. A total 79-fold purification of AchE was achieved by the use of $(NH_4)_2SO_4$ precipitation, gel filtration and chromatography on MAC-Sepharose 4B. The purification shown in Table III yielded enzyme of 2-3 fold greater purity than previously reported (Riov and Jaffe, 1973). Greater yields (2.15 compared to 1.71 units g^{-1} fresh weight) were obtained by Riov and Jaffe (1973) but they achieved a lower specific activity (96 compared to 223 units mg^{-1} protein). The specific activity of the pea AchE was only 37.8 units mg^{-1} protein (Kasturi and Vasantharajan, 1976).

Precipitation with $(NH_4)_2SO_4$ has been used in the purification of cholinesterases (Tzagoloff, 1963; Schwartz, 1967; Fluck and Jaffe, 1974d) and acetylcholinesterases (Kremzner and Wilson, 1963; Leuzinger and Baker, 1967; Dudai, et al., 1972b; Riov and Jaffe, 1973; Kasturi and Vasantharajan, 1976). By the use of gel filtration in the purification protocol, a 5-fold increase in specific activity was achieved (220 units mg^{-1} protein (Figure 6) vs 45 units mg^{-1} protein (Figure 25a)). For this reason it provided a substantial contribution to the purification.

The elution profile for gel filtration on Sepharose 6B in the presence of 0.2M-NaCl contained one peak of AchE activity. This had a migration corresponding to a globular protein mol. wt. of 78 000 (Figure 16). However, the elution profile from gel filtration of

preparations extracted directly with 5% $(NH_4)_2SO_4$ (method B) contained multiple peaks of the activity in the absence of 0.2 M-NaCl (Figure 27). Gel filtration of the mung bean root AchE prepared by a method similar to method A of this study (Riov and Jaffe, 1973) produced one peak of activity which was eluted in the void volume of a column of Sephadex G-200. This corresponded to a globular protein mol. wt. of 200 000. When that enzyme was prepared by the direct extraction of roots with 4% $(NH_4)_2SO_4$ the void volume peak occurred as well as one corresponding to a globular protein mol. wt. of 80 000. In both cases, gel filtration was performed in 20mM-phosphate buffer (pH 7.0) in the absence of NaCl. This apparent inconsistency in gel filtration results could be explained, in part, by analogy with animal AchE. Asymmetric forms of the eel AchE, which contain an elongated structure referred to in the literature as a tail (Dudai, et al., 1973; Reiger, et al., 1973), and the bovine erythrocyte AchE form large aggregates at low (<0.3) ionic strength (Rothenberg and Nachmansohn, 1947; Lawler, 1963; Changeux, 1966; Massoulie and Reiger, 1969; Dudai, et al., 1972a; Crone, 1973). The two peaks in Figure 27 correspond to globular protein mol. wts. of 350 000 and 1 000 000. These may represent 4x and 16x aggregates of the 78 000 mol. wt. species. These high mol. wt. forms would correspond to the peak of AchE activity excluded from Sephadex G-200 (Riov and Jaffe, 1973). An ionic strength dependent equilibrium may exist between these forms such that at a high (0.22) ionic strength all activity would exist in the 78 000 mol. wt. (1X) form. At a lower (0.02) ionic strength some molecules would exist in higher (4X, 16X) aggregation states. Riov and Jaffe (1973) suggested that protease activity in $(NH_4)_2SO_4$ extracts of

roots would account for the presence of the 80 000 mol. wt. form of AchE. They reasoned that most of the protease activity would be removed by the preliminary buffer extract and no degradation of higher mol. wt. forms would occur in the time between $(NH_4)_2SO_4$ extraction of the buffer insoluble material and gel filtration. This proposal is supported by the observation that still lower mol. wt. forms appeared in the elution profile of material extracted directly with 5% $(NH_4)_2SO_4$ (Figure 27) but were absent in the profile of material extracted from the buffer insoluble residue (Figure 5). Proteolytic treatment of eel or torpedo AchE with trypsin or storage of electric tissue in toluene for months (Rothenberg and Nachmansohn, 1947) results in the formation of an active globular form of the enzyme with a sedimentation coefficient of 11 S (Dudai, et al., 1972a; Rosenberry, et al., 1972; Taylor, et al., 1974) and a mol. wt. of 310 000-350 000 (Dudai, et al., 1973; Taylor, et al., 1974; Morrod, 1975) which is incapable of forming aggregates at low ionic strength (Massoulie and Rieger, 1969). Electron microscopy revealed the absence of the "tail" structure in this form (Rieger, et al., 1973).

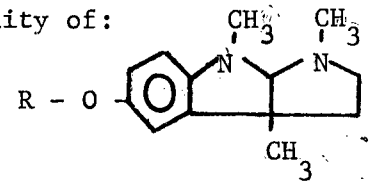
A thorough investigation of the behavior of bean root AchE on columns containing MAC-Sepharose 4B led to its use in the purification protocol. Of all of the conditions tested, those presented in Figure 6 produced the greatest recovery and specific activity. Even when the greatest binding of AchE occurred, the most effective eluant was NaCl. Elution with a NaCl gradient resulted in a single broad peak of activity (Figure 25a). This suggested that the interaction between the ligand and the enzyme was of a non-specific nature. Had the interaction been

specific, a precise ionic strength would have been effective in eluting all of the enzyme (Rosenberry, et al., 1972) and specific elution by decamethonium would have been observed. Use of chromatography with MAC-Sepharose 4B gave a 10-fold purification and this procedure was less effective than in the purification of eel AchE (Dudai, et al., 1972a). The MAC-Sepharose 4B behaved as a true affinity chromatography column against the eel enzyme by the criteria of Cuatrecasas and Anfinsen (1971). The inefficacy of acetylcholine to elute the enzyme (Figure 25b) suggested that the binding was not exclusive to the active center. Furthermore, when there was a delay before the application of NaCl, a portion of the AchE was eluted in the initial volume suggesting a very weak association with the ligand. The observation that decamethonium eluted a smaller quantity of AchE in an equally broad and asymmetric peak (Figure 25c) suggests that the effect of this substance was also nonspecific and related to its contribution to ionic strength rather than its chemical properties. This is supported by the observation that A_{280} values increased gradually in proportion to the ionic strength of decamethonium (Figure 25c).

It is known (Adams and Whittaker, 1950; Wilson and Bergman, 1950; Wilson, 1954; Koelle, 1970; Rosenberry, 1975) that the active center of the AchE from a variety of animal sources contains two primary binding sites — an esteratic site which contains a nucleophilic group specific for the acyl carbonyl and an anionic site which is attracted to the quaternary nitrogen of the choline and also contains a hydrophobic region. Studies with quaternary ammonium, carbamate, and organophosphate inhibitors suggested that the mung bean AchE active center resembles that

of the animal AchE (Riov and Jaffe, 1973). The results of my experiments add further support to this view. The enzyme from P. vulgaris was inhibited by neostigmine (Figure 3) and the inhibition product remained inactive after dialysis suggesting the formation of a carbamyl-enzyme similar to that reported for carbamate inhibition of eel AchE (Wilson, 1954). The bean enzyme was inactivated by DIFP and remained inactive after exhaustive dialysis (Figure 7). Complete characterization of the active center of the enzyme must await further studies using strategies which have proven successful in eel AchE active center investigations.

A major difference between the plant and animal enzymes is in their responses to eserine. Whereas the animal AchE is inhibited by 10^{-5}M-eserine (Augustinsson, 1963; Jackson and Aprison, 1966), the enzyme from roots is inhibited at 10^{-2}M (Riov and Jaffe, 1973; Kasturi and Vasantharaja, 1976). The inhibition of eel AchE by eserine depends on the hydrophobic affinity of:



because, unlike neostigmine, this substance lacks quaternary nitrogen. If the enzyme from roots lacks a substantial hydrophobic component in the anionic binding site, then eserine would not be as effective an inhibitor as of the animal enzyme. This may explain why greater N-methylacridinium concentrations were required to bind the AchE from bean roots than from eel tissue (Figure 23) but would not necessarily answer the question of the ligands apparent nonspecificity. On the other hand, if N-methylacridinium binding was specific for peripheral anionic sites of the eel enzyme, such as those considered to be important

in the binding of decamethonium (Froede and Wilson, 1971; Rosenberry, 1975), then the absence of a corresponding site in the bean root AchE would explain both the non-specific binding of N-methylacridinium and the non-specific elution with decamethonium in both 0.2 and 1.0-M-NaCl. Massoulie and Bon (1976) have pointed out the importance of knowing the binding properties of the enzyme in the application of affinity chromatography to the purification of AchEs. Insufficient information is available pertaining to such properties of both P. vulgaris and Electrophorus AchEs to resolve satisfactorily the inapplicability of this ligand to affinity chromatography of P. vulgaris AchE.

It may be possible to purify the bean AchE by other affinity chromatography ligands. The use of N-acyl-p-aminophenyltrimethylammonium (Dudai, et al., 1972b; Rosenberry, et al., 1972) appears to be especially promising because of the similarity of its structure to that of neostigmine.

The results of electrophoresis in 7% acrylamide gels showed that the AchE was not purified to homogeneity (Figure 8). Other acetylcholinesterases migrate slowly in 7-7.5% acrylamide gels (Dudai, et al., 1972b; Chen, et al., 1974; Steele and Smallman, 1976; Das, et al., 1977). The presence of two bands after gel filtration (Figure 8a) in the vicinity of the enzyme activity suggests that more than one active form of the enzyme may exist in the $(NH_4)_2SO_4$ extracts. The gels were not sliced to detect enzyme activity following gel filtration because the bands were too close together to be resolved. Two bands of AchE activity were observed in acrylamide electrophoregrams of pea root extracts after gel filtration on Sephadex G-200 by Kasturi and Vasantharajan (1976). They did not report the location of these bands. Closely migrating

multiple bands have been observed in the eel (Chen, et al., 1974), house fly head (Steele and Smallman, 1976), and human erythrocyte (Das, et al., 1977) AchEs. Electrophoresis of the globular 11 S form of the eel enzyme appeared as two bands of enzyme activity only one of which was detectable using protein stain (Chen, et al., 1974). An elegant study involving gel filtration, sedimentation, and electrophoresis revealed that the different forms of the fly head AchE were not simply oligomers of the smallest active species (Steele and Smallman, 1976). In the case of the erythrocyte enzyme, a single peak of activity was eluted from Sephadex G-200 in the void volume. This peak was resolved into four unique bands by gel electrophoresis (Das, et al., 1977).

The protein contaminant, R_f=0.69, (Figure 8b), could be a non-active component of the AchE. Das, et al., (1977) observed that the single active peak from gel filtration was separated as one peak of activity on DEAE-cellulose. When this was rechromatographed with smaller mol. wt. protein peaks lacking activity, at least two active AchEs appeared in the DEAE-cellulose elution profile. This supports the existence of either degradation products or a non-AchE matrix which could reassemble with the active enzyme into an AchE having different charge properties. Support of the existence of root AchEs differing in charge properties is apparent from Figure 28. The nature or significance of the major contaminating protein remains undetermined but it could represent a structure similar to the one reported by Das, et al. (1977) because the conditions of gel electrophoresis differed from the conditions of purification. The contaminant may represent a protein not associated with the AchE.

The isoelectric point of AchE was at pH 5.3. The enzyme precipitated with a loss of greater than 90% of the activity at this pH and pH 5.6.

These observations account for low recoveries from thin dextran layers (Figure 13) and the isoelectric focusing column (Figure 14), and explains why I did not use isoelectric focusing in the purification protocol. This property also prevented the successful application of affinity elution chromatography (Scopes, 1977). The peak at pH 7.0 (Figure 14) is difficult to explain. The ΔA_{412} values in the peak resulted from lower A_{412} values in the absence of neostigmine which typify AchE activity.

The autolyzed 11 S globular form of the eel enzyme has a pI of 5.3 (Leuzinger, et al., 1968) or 4.5 (Chen, et al., 1974). Morrod (1975) has reported variable values (pI=3.6-4.9) for the asymmetric forms of that enzyme. Precise values for the forms of the erythrocyte AchE are unavailable but pIs range from 3-5.3 (Das, et al., 1977). The variability of these values probably reflects the instability of all AchEs at their isoelectric points as well as variation in the isoelectric points of the enzyme from different sources. The low pI found in both this and other AchEs suggests the presence of many negatively charged groups. It is likely that both the amino acid and carbohydrate composition of the purified bean AchE will reveal the source of negatively charged groups and explain the anion exchange behavior with the affinity ligand.

The results of the isokinetic sedimentation indicate the presence of a single major sedimenting particle having S=4.2 (Figure 12a). This value used in conjunction with the Stokes radius (4.00 nm) yielded a mol. wt. of 76 000. This approximated the mol. wt. of 78 000 obtained (Figure 16) by assuming that the AchE had a globular structure. An additional calculation yielded the frictional ratio (f/f_0=1.37). This

value is only slightly greater than an average globular protein value of 1.2 (Lehninger, 1970) and provides further evidence as to the globular nature of this enzyme. The accuracy of these values is limited by the assumption that the partial specific volume ($\bar{v}$) of this enzyme is equivalent to that of the eel AchE as reported by Bon, et al., (1973). Their value ($\bar{v}$=0.75cm^{3}g^{-1}) corresponds with an average protein value but differs from other values of 0.714 cm^{3}g^{-1} and 0.793 cm^{3}g^{-1} for the 11 S form of the eel AchE (Bon, et al., 1976) and Lubrol extracted erythrocyte AchE (Beauregard and Roufogalis, 1977), respectively. The high value obtained in the latter case was attributed to the integral association of phospholipid with the enzyme. The use of these values resulted in mol. wts. of 67 000 and 92 000, respectively. The use of the K_{av} value of the AchE obtained during its purification rather than the use of another K_{av} by rechromatography of the purified enzyme may have led to additional errors. However, the accuracy of the calculated mol. wt. is further supported by the similarity of the mol. wt. obtained by SDS gel electrophoresis (77 000, Figure 10) and the glubular protein mol. wt. obtained by gel filtration (78 000, Figure 16), and the observation that K_{av} was reproducible to within 0.01.

An important observation of the sedimentation studies was the presence of a single tritiated particle (Figures 12b and 12c). This particle was established as ^{3}H-DIP-AchE since AchE was stoichiometrically inactivated by ^{3}H-DIFP. The greater sedimentation coefficient of this particle than of the active AchE may have been a function of an altered conformation of the enzyme because a more globular structure would have shown a greater sedimentation coefficient.

Radioactive DIFP labelling strategies have been used to detect catalytic subunits in either AchE purified to homogeneity (Dudai and Silman, 1974; Rosenberry, et al., 1974) or contaminated preparations in which the AchE was protected with butylcholine against activation by non-radioactive DIFP followed, after dialysis, by exclusive AchE labelling with radioactive DIFP (Cohen et al., 1967; Bellhorn, et al., 1970; Berman, 1973). The latter strategy was attempted in this study. Although butylcholine did offer protection against inactivation, the protection was incomplete under the two conditions tested. An alternative method was examined by labeling in the presence or absence of neostigmine. Neostigmine was ineffective in preventing ^{3}H-DIFP labeling (Table V, Figure 12). This may have resulted from incomplete carbamylation of AchE by 125 μM-neostigmine, phosphorylation of some of the carbamylated enzyme, or a more rapid decarbamylation of the enzyme in the presence of DIFP. In any case, neostigmine did not seem to act as an appropriate control for DIFP labeling of AchE. The observations that DIP-AchE sedimented as a single particle corresponding to a slightly more globular form of AchE, that no other tritiated particles were observed in the sedimentation gradients, and that the preparations were free from contaminating esterase activity led to the conclusion that DIP-AchE was a suitable form of the enzyme in which to detect catalytic subunits.

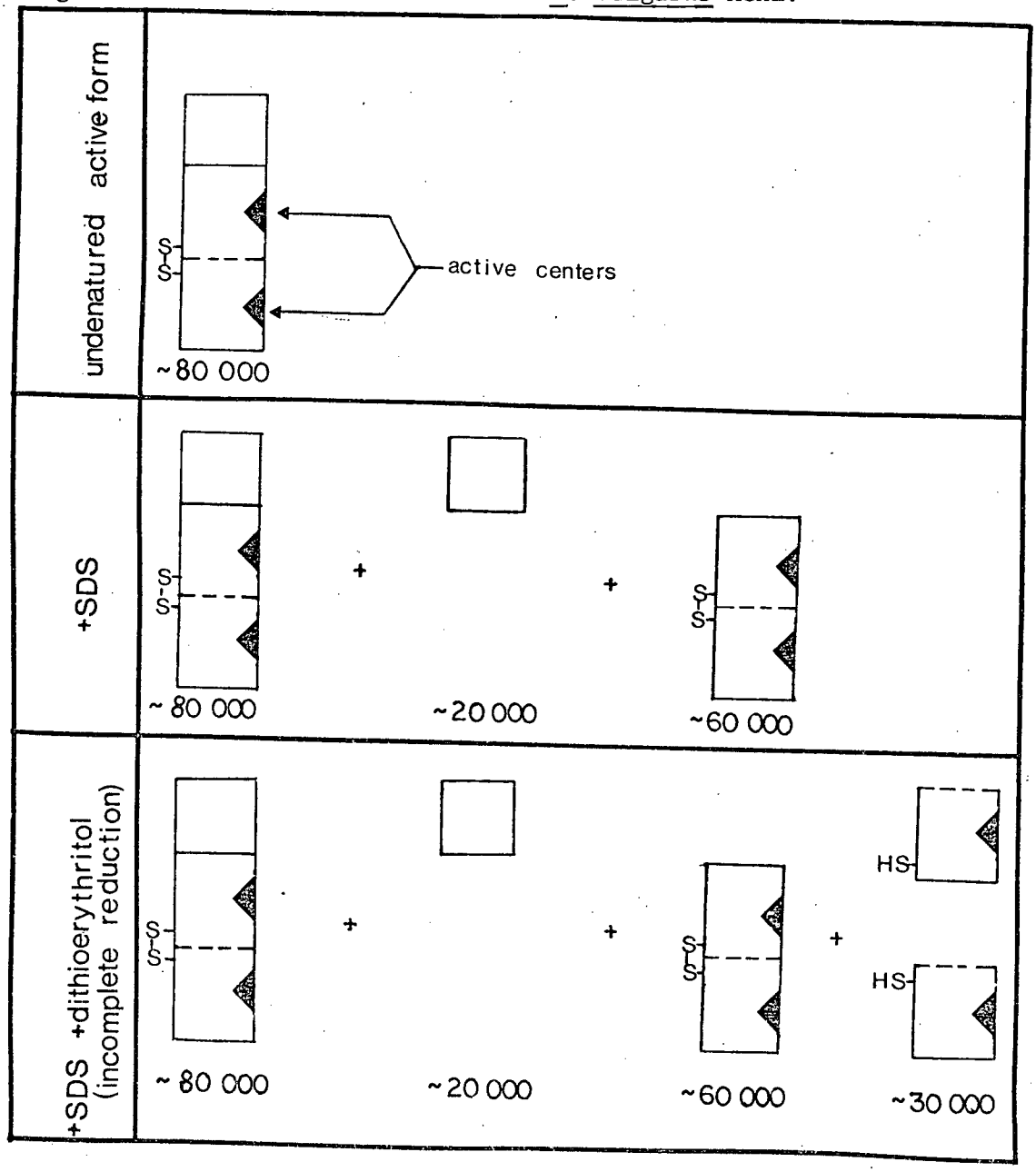
The similarity of the value of 77 000 obtained from SDS gel electrophoresis (Figure 10) and the mol. wt. obtained by gel filtration (Figure 16) suggested that this was a trace of the active form of the AchE undenatured by SDS. However, denaturation yielded a major catalytic

subunit having a mol. wt. of 61 000. Much of the proteinaceous material which centered at 16 000 probably corresponded to the major contaminating protein observed in polyacrylamide gel electrophoresis and isoelectric focusing. The observation that no tritiated peak corresponded to the 16 000 mol. wt. protein (Figures 10a and 11a) supports this statement. The 26 000 mol. wt. shoulder (Figures 10a and 11a) corresponding to a radioactive peak suggested that this represents a second subunit of the AchE. Whether this represented a catalytic subunit is difficult to tell. The observation that, unlike the 61 000 mol. wt. subunit, the radioactivity was slight compared to the protein content suggests that it is not catalytic. From the results of preparations containing protein contaminants it is difficult to assess the significance of the 17 500 mol. wt. radioactive peak, however, it may represent a degradation product of the 61 000 or 26 000 mol. wt. subunit.

With the appearance in reduced samples of radioactivity corresponding to a 30 000 mol. wt. component and a concomitant decrease of activity in the 61 000 dalton component (Figures 10b and 11b) it is tempting to suggest that two 30 000 mol. wt. catalytic subunits are linked by a disulphide bond to form the 61 000 mol. wt. subunit of the active AchE. This observation is tentative because complete reduction was not observed. Conditions must be found in which the 61 000 mol. wt. peak is completely eliminated and replaced by a 30 000 mol. wt. peak. Such conditions were not established in this study.

A tentative subunit structure of the bean AchE can be postulated based on the foregoing information (Figure 32).

Figure 32. Postulated structure of P. vulgaris AchE.

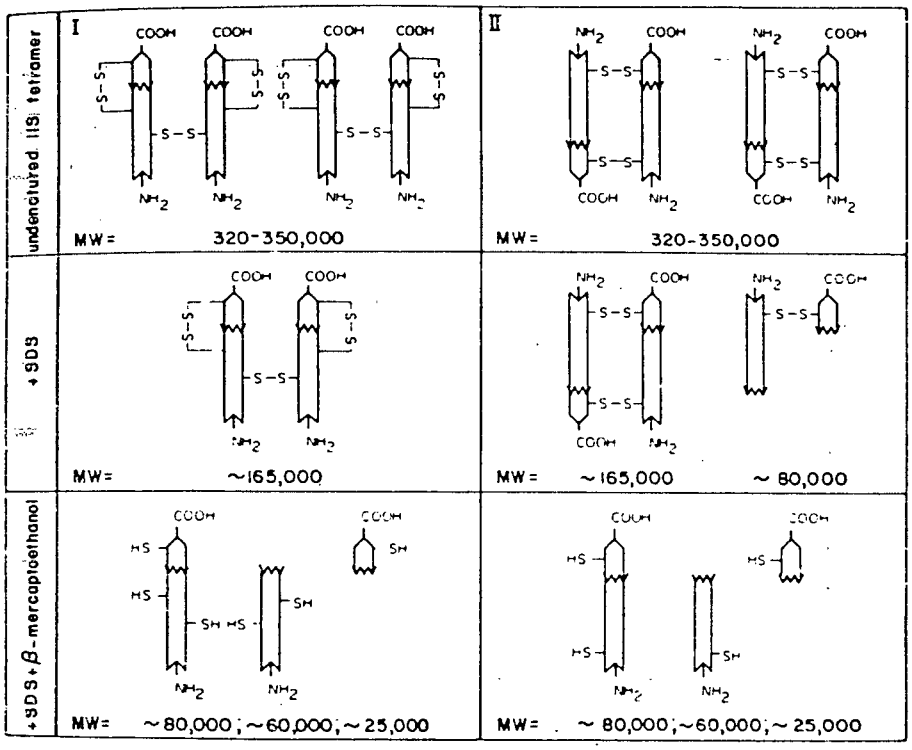


A remarkable similarity exists with the subunit composition of animal AchE. Upon denaturation of the globular (11 S) form of the enzyme, four forms were observed representing tetramer, trimer, and dimer of a monomer having a mol. wt. of 70 000-82 000 in eel (Millar and Grafius, 1970; Dudai and Silman, 1971; Chen, et al., 1974; Dudai and Silman, 1974; Morrod, 1975; Rosenberry, 1977) and 78 000-82 000 in torpedo (Taylor, et al., 1974). When the eel enzyme was reduced in the presence of 2-mercaptoethanol or dithiothreitol, bands appeared having mol. wts. of 75 000-82 000, 50 000-59 000, 25 000-28 000, and 23 000 (Chen, et al., 1974; Dudai and Silman, 1974; Morrod, 1975; Rosenberry, et al., 1974; Rosenberry, 1977). Molecular weight values are suspected to vary because of the glycoprotein nature of the AchE (Rosenberry, 1977). A model has been proposed for the subunit structure of the eel AchE on the basis of these and other observations (Figure 33). The model accounts for the presence of all fragments except the smallest one which is suspected to be a degradation product of the 25 000 mol. wt. form (Morrod, 1975).

In the erythrocyte ghost AchE, the smallest active isolable component has a MW of 200 000 which appears to consist of a dimer with subunits having mol. wts. of 126 000 and 75 000 (Berman, 1973) and containing an integral phospholipid component (Beauregard and Roufogalis, 1977). In the case of the house fly head, the smallest active component had a mol. wt. of approximately 80 000 but no subunits studies have been undertaken (Steele and Smallman, 1976).

Because of the inefficacy of neostigmine in the prevention of DIFP labeling discussed above, the best estimate of catalytic center activity

Figure 33. Schematic model for the 11 S form of eel AchE from Dudai and Silman (1974).



(Florkin and Stoltz, 1973) was obtained by DIFP labeling in the absence of neostigmine (Table V). This value (197 mol of substrate $min^{-1}mol^{-1}$ DIFP) was nearly 5000 times less than the value for the eel AchE (960 000 mol substrate $min^{-1}mol^{-1}$ active center, Rosenberry, 1975). The accuracy of this estimate was limited by the purity of the preparation and the accuracy of the specific activity of ^{3}H-DIFP. A rigorous test of this value awaits the development of a suitable control for non AchE labeling by DIFP.

The maximum theoretical specific activity was estimated from this catalytic center activity by dividing the calculated DIFP binding (mol DIFP mg^{-1} protein by the postulated maximum binding of DIFP assuming the 61 000 mol. wt. subunit contained only one active center per molecule (mol DIFP mol^{-1}AchE). Because the preparations contained 210 units mg^{-1} protein and the binding corresponded to an "equivalent weight" of 950 000, a maximum theoretical specific activity for this enzyme would be 3270 units mg^{-1} Lowry protein. Both a purer preparation and a more accurate or more specific titration of active centers would be required to obtain catalytic subunit equivalent weights and thereby establish whether the 61 000 dalton subunit was the only catalytic subunit.

The low catalytic center activity of this enzyme compared to that of the eel suggests that the requirements for rapid hydrolysis of acetylcholine in these organisms differ tremendously. This is not surprising considering the relatively high quantities and established role of acetylcholine in the electric organ (Florey, 1966) and the low quantities observed in plants (Hartmann and Kilbinger, 1974; White and Pike, 1974).

The Km of P. vulgaris root AchE was 56 μM (Figure 18) for acetylthiocholine. Other Kms have been reported: P. aureus root AchE had a Km of 84 μM (Riov and Jaffe, 1973); Risum sativum root AchE had a Km of 200 μM (Kasturi and Vasantharajan, 1976). The Kms of animal AchEs for acetylcholine vary depending on the ionic strength and range from 90-130 μM in Torpedo and 230-340 μM in Electrophorus (Massoulie and Bon, 1976). No consideration has been given to ionic strength during Km determinations in this or other plant AchEs. Such studies may reveal interesting properties of the enzyme which would facilitate its purification. Furthermore,no rigorous kinetic studies have been performed on any plant cholinesterases.

The response of this enzyme to butyl- and propionylthiocholine (Table VI) was similar to that observed by Riov and Jaffe (1973) although they did not determine the effect of variable concentrations of these substrates on the enzyme activity. In this study, Michaelis-Menten kinetics were not observed for either of these two substrates between 10^{-5} and 10^{-2}M (Figure 17). Unfortunately, a control containing no substrate was not included in this experiment, although extrapolation of the curves to zero substrate suggests a peculiar phenomenon. The response to propionyl- or butylthiocholine suggests that the use of neostigmine in the assay with propionyl- or butylthiocholine was inappropriate. The true zero for these substrates may correspond to ΔA_{412} of approximately 0.1,in which case the actual specific activity for these two substrates in Table VI approaches zero. Riov and Jaffe (1973) have demonstrated that at a substrate concentration of 0.5 mM, neostigmine inhibited the hydrolysis of all three substrates equally. This may not have applied to all substrate concentrations however.

Alternatively,the enzyme may become saturated at a substrate concentration below 10^{-5}M. This seems unlikely in view of the substrate inhibition demonstrated by acetylcholine. Kinetic studies using a different assay method would be required to resolve the problem of substrate affinity. A reevaluation of relative hydrolysis rates and specificity for other esters (Riov and Jaffe, 1973) would be necessary considering the tentative status of hydrolysis rate data for acetyl-, butyl-, and propionyl-thiocholine.

4. Physiological Role of the Acetylcholine/Acetylcholinesterase System.

The possibility that developmental changes are correlated with changes in the status of AchE was suggested earlier in this discussion. Fluck and Jaffe (1974b) observed that light grown mung bean stems have a 10-fold greater extractable AchE activity than etiolated hypocotyls. This suggested the possibility that light may mediate AchE levels as well as acetylcholine levels. The AchE activity in hypocotyls of 5-day old etiolated seedlings exposed to continuous, relatively low intensity white light increased by the same amount over a four day period as the etiolated controls (Figure 29). The increase of activity in etiolated controls was comparable to that observed by Fluck and Jaffe (1974b) for total activity in mung bean roots and by Kasturi and Vasantharajan (1976) for extractable activity in pea roots. Such values show that as the hypocotyl develops,it too increases its AchE activity.

The inefficácy of 2-chlorosulphonic acid, an ethylene generating growth regulator applied in concentrations which were effective in inducing the "triple response" symptoms (Neljubow, 1901), in altering AchE levels (Figure 29) suggests that any role that AchE may have in the

hook opening response is not affected by ethylene. This could mean that the inhibitory effect of acetylcholine on ethylene generation and subsequent hook closure (Parups, 1976) is independent of the AchE level in the tissues, although no conclusive evidence has been obtained.

Kinetin at 10^{-4}M inhibits hook opening and GA at 10^{-3}M enhances opening (Kang and Ray, 1969). It is interesting that these regulators have opposite and statistically significant effects on AchE of etiolated P. vulgaris hypocotyls (Figure 30). The time course involved in this effect is substantially longer than the 8-12 hours required for the initiation of hook opening in excised hooks. Based on the time course of the response, any regulation of acetylcholine in the hook opening response would be attributed to constitutive enzyme activity and the variation in AchE activity more likely would be linked with a longer term developmental change. Considering the observation that AchE activity increased with the age of the tissue, it is conceivable that AchE may be involved in the actual process of aging itself. The observation that kinetin, a growth regulator active in counteracting many senescent processes (Sacher, 1973) reduced AchE activity in hypocotyls (Figure 30) supports this idea.

Even though all growth regulators studied in this project were tested at concentrations in which they are effective in inducing hook opening responses, the possibility that other concentrations may exert different effects cannot be discounted, nor can the possibility that the concentrations effective in the excised hook opening assay differ from optimal concentrations of sprayed applications to intact plants.

Parups (1976) observed that acetylcholine partially prevented the IAA-promoted delay of hook opening and inhibited IAA-induced ethylene production. By these actions, acetylcholine appears to act as an antagonist of IAA thereby preventing ethylene from becoming available to induce hook closure. Parups (1976) postulated further that the action of acetylcholine was in mimicking the effect of red light on IAA concentration and ethylene production. On this basis it would be likely that at some concentration, acetylcholine alone would enhance hook opening. This was not observed between the concentrations of 10^{-9} and 10^{-3}M (Figure 31) nor was any elongation response induced by 10^{-6} M acetylcholine.

In view of the increasing number of observations of acetylcholine effects on assorted plant processes (Dekhuijzen, 1973; Grerrin, et al., 1973; Penel, et al., 1976; Sharna et al., 1977) and its endogenous existence (Jaffe, 1970; Hartmann and Kilbinger, 1974), it would not be surprising to find that more plant responses or developmental changes than secondary root formation were mediated by acetylcholine. The results of this portion of the study are inconclusive but point the way to an obvious area of study in developmental biology.

E. SUMMARY

Acetylcholinesterase activity was studied to identify, purify and characterize the enzyme which may be responsible for regulating acetylcholine levels in plant tissue and to assess the role of the enzyme activity in plant tissues.

A unique AchE was identified in etiolated P. vulgaris roots and tentatively identified in the hypocotyl by use of a colorimetric assay

which included the cholinesterase inhibitor neostigmine as a control. A form of the AchE was extracted with 5% $(NH_4)_2SO_4$ from a buffer in soluble residue of root tissue and purified to a specific activity of 210±20 units mg^{-1} protein-greater than twice the previously reported maximum. This preparation contained one major contaminating protein which may or may not have been associated with the active AchE but did not contain any other esterase activity. The requirement of high purity has been established as a necessity for the distinction between cholinesterase and acetylcholinesterase activities in plant tissues.

An unidentified dialyzable inhibitor of AchE activity was located in buffer homogenates of both root and hypocotyl tissues and the possibility of a non-dialyzable inhibitor was proposed. The presence of inhibitors illustrated the need for a different assay to establish quantitative levels of enzyme activity in situ.

The existence of other forms of AchE including a 4x and possibly a 16x aggregate of the purified AchE was demonstrated and it was suggested that both aggregates and iso enzymes might exist.

The major fraction of the hypocotyl enzyme was located in the cell wall.

Physical and catalytic properties of the root AchE were studied and are summarized in Table IX. Many of these properties are similar to those of the AchE from various animal sources. A subunit structure has been tentatively proposed on the basis of results from SDS gel electrophoresis and K_{av} measurements. Of the information obtained in this and two previous studies of plant AchEs, it was concluded that although catalytic properties of this enzyme differ between plants and animals, structural

Table IX. Summary of Physical Properties of P. vulgaris AchE

Physical parameter (Method)	Value
$S_{20\ w}$ (sedimentation velocity)	4.2 ± 0.1 S
Mol. wt. (gel filtration and sedimentation velocity) *	76 000 ± 2 000
Mol. wt. (SDS gel electrophoresis)	77 000 ± 2 000
Subunit mol. wts. (SDS gel electrophoresis)	61 000 ± 2 000 (2 X 30 000 ± 1 000)
	26 000 ± 2 000
Stokes radius (gel filtration)	4.00 nm
f/f_0 *	1.37
Catalytic center activity (DIFP titration)	197 ± 5 $mol\ min^{-1}\ mol^{-1}$ substrate active center
Isoelectric point	5.3 ± 0.1

* $\bar{v} = 0.75\ cm^3 g^{-1}$ (assumed)

properties are remarkably similar.

The conclusion that this enzyme is involved in the hydrolysis of acetylcholine to the exclusion of other esters *in situ* cannot be drawn from existing data. It was suggested that AchE may not be important in the short term regulation of acetylcholine in such responses as hook opening but may be involved in regulation of acetylcholine levels over longer time periods and may thus be important for normal plant development.

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CHAPTER II

THE ABSENCE OF NUCLEOPHILIC SITES IN THE CELL WALLS OF ETIOLATED PHASEOLUS VULGARIS L. HYPOCOTYLS AND ITS RELATION TO CELL ELONGATION

A. INTRODUCTION

Plant cell elongation has been a topic of interest for decades and has been extensively reviewed (Heyn, 1940; Setterfield and Bayley, 1961; Frey-Wyssling, 1962; Wilson, 1964; Lockhart, 1965; Morre and Eisinger, 1968; Cleland, 1971; Preston, 1974). It is generally agreed that 1) turgor pressure is a driving force for cell extension, 2) cell extension involves changes in the viscoelastic properties of the primary cell wall resulting in reduced cell wall resistance to stress, and 3) deposition of cell wall polymers is required for continuation of cell extension. However, the arrangement of cell wall polymers before, during, and after cell extension, the cause and the molecular location of the viscoelastic changes, and the controls which operate upon cell extension remain uncharacterized.

An understanding of the arrangement and linkages of cell wall polymers is necessary for characterization of the structural changes which occur during cell wall extension. The primary cell wall of higher plants consists of cellulosic microfibrils surrounded by an apparently amorphous matrix of hemicelluloses, pectic acids, and both enzymatic and structural proteins. Our present understanding of the primary cell wall structure is based upon observations using such techniques as polar and

interference microscopy (Ruge, 1937; Diehl, et al., 1939), x-ray diffraction (Meyer and Misch, 1936; Berkeley and Kerr, 1946), electron microscopy (Frey-Wyssling, et al., 1948; Heyn, 1966), histochemistry (Reis and Roland, 1974), chemical (Selvendran, et al., 1975; Monro, et al., 1976) and enzymatic (Karr and Albersheim, 1970) degradation, and chemical analysis (Bauer, et al., 1973; Talmadge, et al., 1973).

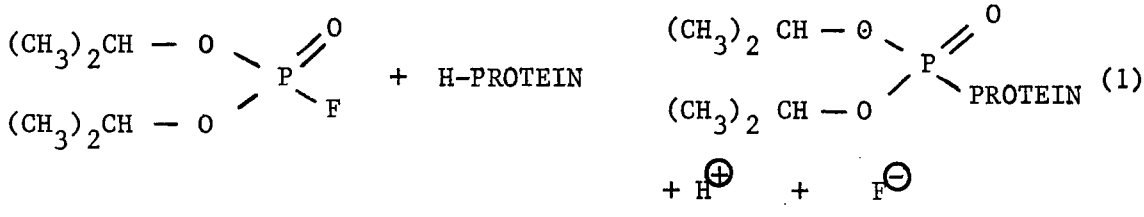
In lateral primary cell walls of many cell types (Roelofsen, 1951; Wardrop, 1956; Setterfield and Bayley, 1958; Preston, 1974) the orientation of microfibrils on the inner wall surface is perpendicular to the major cell axis becoming parallel as the cell extends and more material is added to the existing cell wall. Thus, according to the multinet growth hypothesis (Roelofsen and Houwink, 1951), movement of the microfibrils with respect to the matrix comprises a major event during cell extension. But microfibrillar movement depends upon a recorganization of the matrix accompanying reduced cell wall resistance to stress (Barnicki-Garcia, 1973; Preston, 1974). It is postulated that bonds either are broken, newly formed, or both broken and reformed during cell wall extension.

Recently, specific linkages have been examined with respect to their susceptibility to cleavage or formation under conditions which increase, decrease, or terminate cell wall extension. These linkages include hydrogen bonding involving the xyloglucan component of hemicellulose (Labavitch and Ray, 1974), arabinosyl-hydroxyproline (Lamport, 1965; Lamport, 1970) and galactosyl-serine (Lamport, et al., 1973; Cho and Chrispeels, 1976) of the cell wall glycoprotein, β-1, 4-glucosyl linkages in the cellulose (Wong, et al., 1977), and ferulic

acid-polysaccharide esters (Hartley, et al., 1976).

Linkages involved in the glycosylation of cell wall protein are formed during biosynthesis of cell wall components before export and incorporation into the cell wall (Chrispeels, 1976). However, if the cross-linking of cell wall glycoprotein that is believed to accompany the cessation of elongation (Lamport, 1965; Sadava, et al., 1973) occurs between pre-existing cell wall protein and newly exported cell wall polysaccharides, then glycosylation would also occur in the cell wall. The probability of such a modification occurring in the cell wall would be supported if there were some sites in the glycoprotein which were not glycosylated in young actively extending cell walls but were found glycosylated in older, fully extended cell walls. Positive evidence for such sites would be obtained if the sites were reactive toward an artificial modification reagent and if the reagent could be incorporated into preparations of actively elongating cell walls. A failure to detect binding would suggest the absence of suitably reactive sites. Diisopropylfluorophosphate (DIFP) could act as such a reagent.

DIFP has been widely used in protein modification and enzyme inhibition studies (Cohen, et al., 1967). In the reaction:



(1)

the electrophilic phosphorus reacts with a nucleophilic group of the protein leading to a departure of $F^{\ominus}$ and formation of a diisopropyl-phosphoryl-(DIP-)protein. A major product of partial acid hydrolysis of most DIP-proteins is phosphoserine (Schaffer, et al., 1953; Schaffer,

et al., 1954; Cohen, et al., 1967) indicating that the serine hydroxyl group is the nucleophile in reaction (1). As hydrolysis procedes, the phosphate ester is also cleaved yielding orthophosphoric acid. In the case of DIFP inhibition of acid phosphomonoesterase, no phosphoserine was recovered, indicating that other nucleophilic sites could react with DIFP (Greenberg and Nachmansohn, 1965).

The purpose of this study was to examine the cell walls of hypocotyls of the bush bean (Phaseolus vulgaris L.) for the presence of serine residues which were reactive by nucleophilic substitution. The bean hypocotyl offers a developmental continuum of elongation stages from the apical region consisting of non-elongated cells to the basal region consisting of elongated cells (Bailey and Kauss, 1974). By sampling regions along this continuum, the application of exogenous stimuli to induce or terminate elongation is avoided. The growth patterns of the cell types present conform to the multinet growth hypothesis with the exception of the epidermal cells (Bayley, et al., 1957). Though the glycosyl linkages to cell wall protein have not been examined in P. vulgaris, compositional studies of the primary cell walls derived from this plant (Wilder and Albersheim, 1973) suggest that it is similar to other members of the Dicotyledonae whose glycosyl linkages have been studied.

In this study, phosphoserine was analyzed following the reaction of DIFP with a) serine, b) α-chymotrypsin, and c) cell walls of elongating and fully elongated cells from etiolated bush bean hypocotyls. A comparison was made between phosphoserine recovered from cell walls labeled with ^{32}P-DIFP and cell walls labeled with ^{32}P-DIFP following a

pretreatment with non-radioactive DIFP to correct for non-specific labeling or adsorption of the radioisotope. This comparison led to the identification of serine labeling in cell wall proteins under conditions which yielded a nearly complete modification of serine in the α-chymotrypsin active center. Reactivity of α-chymotrypsin and the cell walls toward a spin-labeled analogue of DIFP (2,2,6,6-hydroxytetramethylpiperidinooxyl monoethylfluorophosphate (HTMFP)) was also examined.

B. MATERIALS AND METHODS

1. Chemicals

Supplies were obtained from sources as indicated: DIFP: Aldrich Chemical Co., Milwaukee, Wi.; ^{32}P-orthophosphoric acid and ^{32}P-DIFP: Amersham/Searle, Arlington Heights, Il.; HTMFP: Syva Assoc. Inc., Palo Alto, Ca.; cellulose powder: W. & R. Balston Ltd., England; ethylene glycol monomethyl ether (methylcellosolve) and ninhydrin: Pierce Chemical Co., Rockford, Il.; Folin-Ciocalteu phenol reagent: Harleco, Philadelphia, Pa.; trichloroacetic acid (TCA), p-terphenyl, 1,4-bis-2-(5-phenyloxazolyl)-benzene and National Bureau of Standards calibrated HCl: Fisher Scientific Co., Fairlawn, N.J.; isopropanol, dioxane, and naphthalene: Mallinckrodt Chemical Works, St. Louis, Mo.; Beckman Amino Acid Calibration Mixture Type I: Beckman Instruments Inc., Spinco Div., Palo Alto, Ca.; phosphoserine: California Biochemical Corp., Los Angeles, Ca.; N-acetyl tyrosine ethyl ester: Sigma Chemical Co., St. Louis, Mo.; α-chymotrypsin: Worthington Biochemical Corp.; Freehold, N.J. All other chemicals were obtained locally. "Baker Analyzed" grade (J. T. Baker Chemical Co., Phillipsburg, N.J.) was used when available.

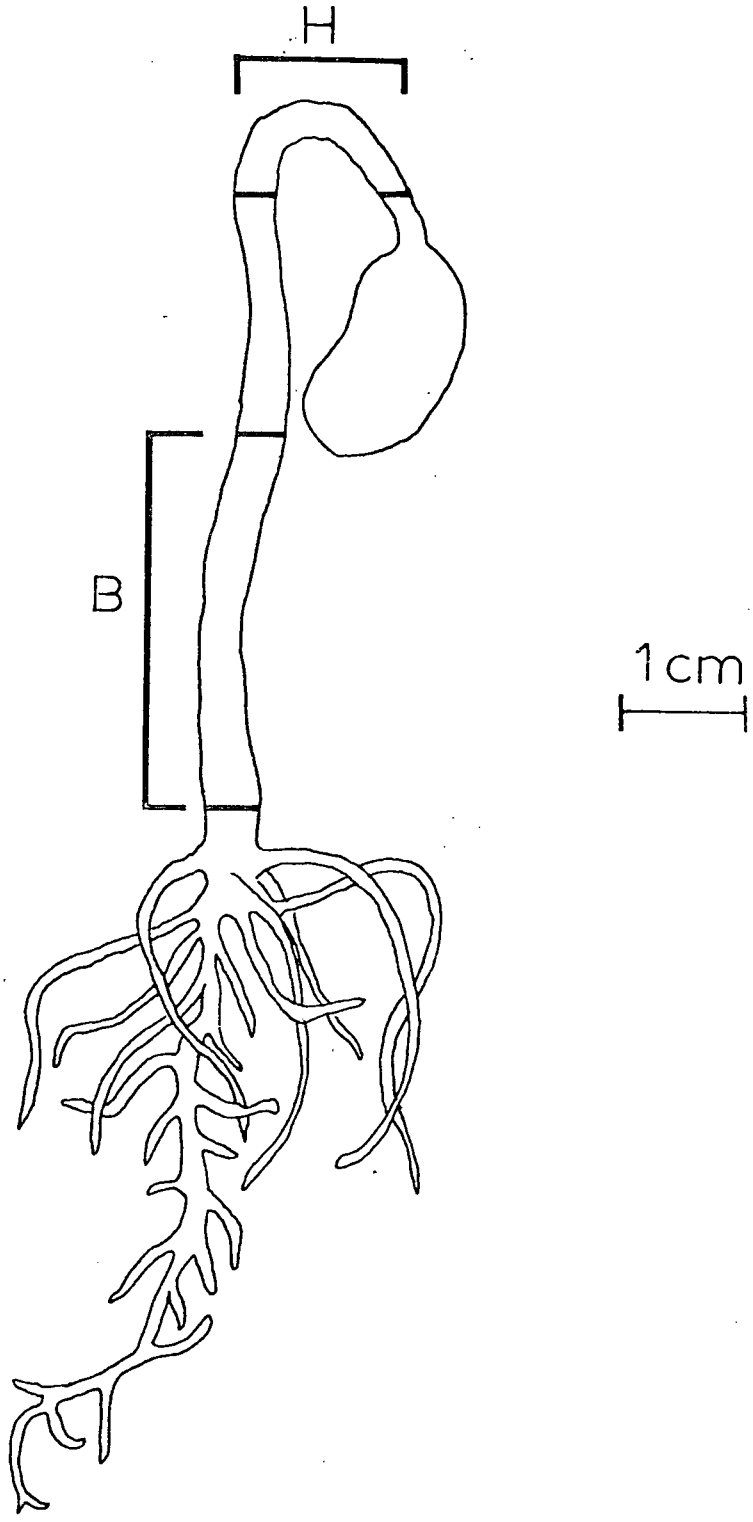
2. Plant Material

Bush bean (Phaseolus vulgaris L. var. Top Crop Green Pod) seeds were obtained from Buckerfields Ltd., Vancouver, B.C. They were grown in vermiculite for 7 days in a dark wooden cabinet at room temperature. Hypocotyls were separated from the cotyledons and roots. Segments (1-5 cm) of either entire hypocotyls, hook regions, or basal regions (Figure 1) were prepared for cell wall extractions.

3. Cell Wall Extraction

Hypocotyl tissue (50g) was ground toaa fine powder in liquid nitrogen (8-10 min). The frozen powder was left to melt at 0°C. The following extraction was carried out at 0-4°C. The homogenate was suspended in 500 ml of deionized water and allowed to settle until 2 layers appeared (10-20 min). The upper layer was transferred to centrifuge bottles. The lower layer was resuspended and the settling procedure was repeated twice. The cell wall fragments were collected from the pooled upper layers by centrifugation at 15 000 g for 10 min. The pellet was resuspended in 200 ml of deionized water. This suspension contained less than 2% of intact cells as determined by light microscopy. The suspension was treated with a Blackstone Ultrasonic Probe for 2 min at 200±50 W to release attached cytoplasmic contaminants. The fragments were collected by centrifugation at 10 000 g for 15 min, resuspended in water and treated again with the ultrasonic probe. This procedure was repeated until the cell walls were free from cytoplasmic contaminants as determined by phase contrast microscopy. Four washings were usually enough to achieve the desired purity. Cell walls were lyophilized and stored at 0°C. Yields varied from 1-4 mg dry cell wall per g of hypocotyl.

Figure 1. Diagram of an etiolated bean hypocotyl illustrating the regions from which cell walls were extracted. H - hook region; B - basal region.



4. Reaction of Serine with DIFP

The reaction of serine with DIFP was established by addition of 1 ml of 2% (w/v) DIFP in CaO-dried isopropanol to 1 μmol of serine in 350 μl of 0.1 M-sodium phsphate buffer, pH 7.3. The mixture was incubated for 45 min at room temperature, and 0.27, 0.45, or 1.35 ml of 12 M-HCl was added to initiate hydrolysis. Tubes were hydrolyzed *in vacuo* for periods ranging from 2 to 30 h. Hydrolyzates were dried in a dessicator over KOH pellets and resuspended in 500 μl of deionized water. Each tube was prepared in duplicate.

Two hundred μl of each sample and 200 μl of Beckman Amino Acid Calibration Mixture Type I, containing 20 nmol of the common protein amino acids, were applied to a 0.9 x 57 cm column on a Beckman Amino Acid Analyzer Model 120 C. Amino acids were separated and phosphoserine was analyzed according to the method of Spackman, *et al.*, (1958) using asparatic acid as an internal standard.

5. Reaction of Alpha-chymotrypsin with DIFP

The reaction of α-chymotrypsin with radioactive and non-radioactive DIFP was performed by a modification of the method of Schaffer, *et al.* (1954). A typical reaction mixture contained 50 μl of a ^{32}P-DIFP (470 Ci/mg) solution (0.96 mg/ml of CaO-dried isopropanol), 1 ml of an α-chymotrypsin solution (5 mg/ml of 0.05M-sodium phosphate buffer, pH, 7.3), and 10 μl of a 2% (v/x) solution of DIFP in CaO-dried isopropanol. A reaction control contained 1 ml of α-chymotrypsin solution (5 mg/ml of 0.05M-sodium phosphate buffer, pH 7.3) and 10 μl of a 10% (w/v) solution of DIFP in CaO-dried isopropanol and was incubated for

90 min at room temperature before 50 µl of a ^{32}P-DIFP (470 µCi/mg) solution (0.96 mg/ml of CaO-dried isopropanol) was added.

Tubes were incubated for 45 min at room temperature; excess ^{32}P-DIFP was removed either by dialysis against deionized water for 36 h at 4°C, or by precipitation of the protein with 20% TCA, centrifugation at 600 g for 1 min, and seven washes with water. Labeled protein was lyophilized and stored at 0°C.

In one experiment, the temperature ana reaction time were varied to test for conditions yielding optimal inhibition of enzymatic activity by DIFP. Alpha-chymotrypsin was allowed to react with DIFP either by the method of Cohen, et al., (1967) or Schaffer, et al. (1954). Tubes were placed on ice until α-chymotrypsin assays began.

6. Reaction of Cell Walls with DIFP

One ml of 0.05 M-sodium phosphate buffer, pH 7.5, was added to 30 mg of cell walls; radioactive and non-radioactive DIFP were added to this suspension in amounts described in the previous section. Reactions were carried out as described above. Excess reagent was removed either by dialysis against deionized water for 36 h at 4°C or by centrifugation at 600 g for 1 min and resuspension in deionized water repeated until no radioactivity was detected in the supernatant (10 times). Cell walls were lyophilized and stored at 0°C.

7. Recovery of Phosphoserine

To confirm and quantitate the extent of diisopropylphosphorylation of serine by DIFP, samples of the derivatized α-chymotrypsin were subjected to serial hydrolysis. The lyophilized protein (3.00 mg)

was dissolved in 0.4 ml of 2 M-HCl and hydrolyzed for 6, 12, 18, 21, and 24 h at 100°C *in vacuo*. Derivatized cell walls were hydrolyzed in 2 M-HCl for 21 hr at 100°C *in vacuo*. The acid was removed *in vacuo* over KOH pellets and silica gel. Each hydrolyzate was dissolved in 0.5 ml deionized water, 2 µl of dansyl hydroxide was added as a fluorescent internal standard, and the sample was applied as a spot to a sheet of Whatman 3MM chromatography paper. Phosphoserine, orthophosphoric acid and a colored marker (DNP-aspartic acid) were applied beside the sample. The mixture was resolved by electrophoresis for 25-30 min at pH 3.5 at 50 V/cm (Ambler, 1963). Phosphoserine and orthophosphoric acid standards were detected by cadmium-ninhydrin reagent (Heilmann, *et al.*, 1957) and ammonium molybdate-ascorbic acid reagent (Ames, 1966), respectively. Mobilities of standard phosphoserine and orthophosphate were determined with reference to DNP-aspartate and dansyl hydroxide. Electrophoregrams were cut into strips and scanned for ^{32}P activity using an Actigraph II Model 1025 strip scanner (Nuclear Chicago) coupled to a Nuclear Chicago Model CR8416 chart recorder. All radioactive compounds had mobilities corresponding to either phosphoserine or orthophosphate standards. Samples having mobilities corresponding to these standards were cut from electrophoregrams and finely minced with scissors for liquid scintillation counting.

Two hydrolyzates were separated by ion exchange chromatography as described for the reaction of serine with DIFP. On both occasions, the total activity recovered in phsophoserine and orthophosphate was within 4% of the corresponding activity recovered following high voltage paper electrophoresis.

8. Determination of Radioactivity

10 ml of diozane-based scintillation fluid (Bray, 1960) were added to aqueous solution, suspensions, lyophilized solids or finely cut pieces of Whatman 3MM paper. Samples were counted in an Isocap 300 liquid scintillation spectrometer (Nuclear Chicago) for 20 min with an 800 K cpm termination at a window of 25 to 1700 Kev. A $^{32}_{3}P$ quench curve was prepared using the external standard ratio method (Wang and Willis, 1965) by the addition, in triplicate, of 0.00, 0.15, 0.30, 0.50, 1.00, and 3.00 ml of deionized water to 10 µl of undiluted ^{32}P-phosphate solution in 10 ml of Bray's solution. All activities were below the coincidence counting range of the spectrometer. Activities of ^{32}P-DIFP stock solutions were determined to correct for incomplete transfer of ^{32}P-DIFP to isopropanol. Thus, the quench curve ordinate was cpm/dpm calculated for ^{32}P-DIFP and sample activities were expressed as a mole fraction of original ^{32}P-DIFP. Background was counted in triplicate before each series of samples and subtracted from sample counts prior to activity determinations. All determinations were performed in triplicate on at least two preparations.

9. Calculations

A correction was made for non-specific ^{32}P-DIFP labeling by subtracting mean values of DIFP-pretreated groups from mean values of treatments lacking DIFP pretreatment. These differences were tested for statistical significance using the Student t-distribution and weighted variance for unequal sample sizes. All corrected values for ^{32}P recovery were significant at $\alpha=0.01$ unless indicated otherwise.

10. Protein Determination

Cell walls were extracted in 1 M-KOH for 30 min in a boiling water bath. The mixture was diluted ten-fold, shaken, and allowed to settle. The protein in the supernatant was determined by the method of Lowry, et al., (1951) as modified by Eggstein and Kreutz (1955). The residue of extracted cell walls washed twice with deionized water showed no protein by the qualitative microbiuret assay (Goa, 1953). All determinations were performed in triplicate on at least two cell wall preparations.

11. Assay of Alpha-chymotrypsin Activity

Activity of α-chymotrypsin was determined by a modification of the method of Cohen, et al., (1967) using a Radiometer pH-stat. Twenty ml of 0.05 M-KCl containing 22.5 mg of N-acetyl tyrosine ethyl ester were equilibrated at 30°C. One hundred µl of the appropriate dilution of native and DIP-α-chymotrypsin in 0.05 M-Tris HCl, pH 7.5, were added. The volume of 0.01 M-NaOH required to titrate liberated acetic acid to pH 7.5 was automatically recorded for 1-2 min. Activities were determined from initial slopes and recorded as mol of substrate hydrolyzed $min^{-1} mg^{-1}$ (units) of α-chymotrypsin. Titrant molarity was confirmed by titration against Nations Bureau of Standards-calibrated 1 M-HCl.

12. Spin Labeling

The spin labeling method was based on procedures described by Schaffer, et al., (1954) for eel cholinesterase diisopropylphosphorylation with modifications relevant to the use of the spin label 1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmethylphosphonofluoridate (Morrisett, et al., 1969).

Fifty µl of 5% (w/v) HTMFP in benezene were added to 3 mg of cell walls or Whatman cellulose powder in 1 ml of 0.1 M-sodium phosphate buffer, pH 7.3. Other tubes contained 50 µl of HTMFP alone in buffer and 50 µl of HTMFP with 3 mg of cell walls that had been pre-treated with 100 µl of 2% (w/v) DIFP in CaO-dried isopropanol for 45 min then washed with approximately 1 ml of buffer and centrifuged at 600 g for 1 min at room temperature. This washing procedure was repeated 12 times.

The mixtures were shaken and incubated at room temperature for 1 h. Excess spin label was removed by centrifugation at 600 g for 1 min and resuspension in deionized water 12 times until no spin label was detected in the wash or diffusate, respectively.

Spectra of suspensions (approximately 6 mg/ml) were recorded at room temperature in a low temperature aqueous solution quartz cell (James F. Scalon, Solvang, Ca.) using a Varian E-3 electron spin resonance spectrometer (Department of Chemistry, University of British Columbia) with an H field range of 3435 - 3535 Gauss and detector power of 5.00 mW at 9.523 GHz.

Alpha-chymotrypsin was spin labeled by the same method. Excess reagent was removed by the method described for the removal of excess ^{32}P-DIFP from labeled α-chymotrypsin.

C. RESULTS

1. Modification of Serine with DIFP

Table I shows the recoveries of phosphoserine from hydrolyzates of mixtures following the reaction of serine with DIFP. Recovery depended upon hydrolytic conditions. A maximum of 1.1% of the original

Table I. Phosphoserine recovered following the reaction between serine and DIFP and serial hydrolysis with 2, 3, or 6 M-HCl. Values are means of duplicate reactions and are expressed as a percentage of serine in the initial reaction mixture. N.D. denotes values not determined.

HCl concentration	Duration of hydrolysis (hr) 2	4	8	15	24	30
2 M	N.D.	N.D.	N.D.	1.0	0.0	1.1
3 M	N.D.	Trace	0.1	0.3	N.D.	N.D.
6 M	0.7	0.8	0.1	N.D.	N.D.	N.D.

serine was recovered as phosphoserine. No phosphoserine was detected in the serine solution used for the reaction. Only phosphoserine and serine were observed in any analysis of ninhydrin positive substances. The presence of isopropanol in the reaction mixture did not effect chromatography of products. Unreacted serine was not measured after hydrolysis because, based on phsophoserine recovery, the quantity of DIP-serine and thus the difference between serine quantities before and after diisopropylphosphorylation and hydrolysis was smaller than the average error in an anàlysis.

2. Modification of Alpha-chymotrypsin with DIFP

Alpha-chymotrypsin was treated with DIFP and assayed for its ability to hydrolyze N-acetyl tyrosine ethyl ester. The activity was inhibited by DIFP under both reaction conditions tested and inhibition was essentially complete after 20 min at 30°C (Table II). The inhibited enzyme was used as a test system to measure recovery of phosphoserine following diisopropylphosphyorylation with ^{32}P-DIFP.

There were 0.583 mol of phosphorus-32 recovered per mol of α-chymotrypsin, regardless of the method used to remove excess ^{32}P-DIFP from ^{32}P-DIP-α-chymotrypsin (Table III). Under reaction conditions which result in 68.2% inhibition of α-chymotrypsin (Table II), 58.3% of the α-chymotrypsin was labeled with ^{32}P. Thus, the molar ratio of bound ^{32}P-DIFP to active centers inhibited by DIFP is 0.855. This is in close agreement with the value of 1.00 established by Jansen, et al. (1950.

Table II. Activity of native and DIFP-inhibited α-chymotrypsin measured by hydrolysis of the synthetic substrate, N-acetyl tyrosine ethyl ester. Values represent means of duplicate assays.

Inhibition reaction conditions	Specific activity (units mg^{-1} α-chymotrypsin)	% inhibition
Native enzyme (no inhibition)	216.0	0.0
Enzyme after reaction with DIFP for 20 min at 30°C	1.2	99.3
Enzyme after reaction with DIFP for 45 min at 24°C	64.2	68.2

Table III. Recovery of ^{32}P activity from DIFP pretreated and non-pretreated ^{32}P-DIP-α-chymotrypsin. Excess ^{32}P-DIFP was removed from ^{32}P-DIP-α-chymotrypsin by either dialysis or 20% TCA precipitation of the protein. Phosphorus-32 recoveries are expressed as a mole fraction of α-chymotrypsin ± S.E.M.

Method of ^{32}P-DIFP removal	DIFP pretreatment (mol ^{32}P mol^{-1} α-chymotrypsin)	No DIFP pretreatment	Corrected value
Dialysis	0.116 ± 0.001	0.699 ± 0.028	0.583
Protein precipitation	0.003 ± 0.002	0.586 ± 0.056	0.583

3. Phosphoserine Recovery from ^{32}P-DIP-α-chymotrypsin

The products of the partial hydrolysis of diisopropylphosphoryl enzymes — phosphorserine and orthophosphoric acid (Schaffer, et al., 1953; Schaffer, et ala., 1954) — were assayed for radioactivity after serial hydrolysis of ^{32}P-DIP-α-chymotrypsin to establish conditions for quantitative or optimal recovery of ^{32}P from cell walls treated with ^{32}P-DIFP. When hydrolysis products were separated by high voltage paper electrophoresis, the total activity recovered from electrophoregrams varied from 14 to 38% (Table IV). Optimal but incomplete recovery of phosphoserine occurred after 12 h of hydrolysis. Phosphate recovery increased with hydrolysis time and total phosphorus-32 recovery increased after 18 h. The molar ratio of phosphoserine to α-chymotrypsin was at least one order of magnitude less than the corresponding ratio for DIP-α-chymotrypsin from Table III. If I assumed that unrecovered activity was distributed proportionately in phosphoserine and phosphate, then the corrected values presented in Table IV were obtained. Each sample was corrected independently. These values are consistently less than half of the corresponding molar ratio for DIP-α-chymotrypsin from Table III. This result is in agreement with those of others (Schaffer, et al., 1953; Schaffer, et al., 1954).

4. Modification of Cell Walls with DIFP

Cell walls isolated from entire hypocotyls of etiolated P. vulgaris were not labeled by ^{32}P-DIFP after correcting for non-specific binding of ^{32}P-DIFP, when excess ^{32}P-DIFP was removed by dialysis (Table V). Cell walls isolated from hook and basal regions of hypocotyls of etiolated P. vulgaris contained 5.4 and 3.4 pmol of ^{32}P mg^{-1} of dry

Table IV. Recovery of ^{32}P from electrophoregrams after serial hydrolysis of DIP-α-chymotrypsin. Phosphoserine and orthophosphate recoveries are expressed as molar ratios of α-chymotrypsin. Total ^{32}P recoveries are expressed as percentages of the ^{32}P activities in DIP-α-chymotrypsin before hydrolysis. Phosphoserine recoveries are corrected for loss during hydrolysis and electrophoresis reflected in individual total recoveries. Values are means of duplicate analyses.

		Duration of hydrolysis (h)				
		6	12	18	21	24
Phosphoserine Recovery (mol phosphoserine/ mol α-chymotrypsin)	DIFP pretreatment	0.0001	0.0006	0.0002	0.0001	0.0001
	No DIFP pretreatment	0.0382	0.0553	0.0432	0.0387	0.0450
	Corrected value	0.0381	0.0547	0.0430	0.0386	0.0449
Orthophosphate Recovery (mol orthophosphate/ mol α-chymotrypsin)	DIFP pretreatment	0.0004	0.0035	0.0009	0.0027	0.0001
	No DIFP pretreatment	0.0281	0.0680	0.0641	0.0770	0.0930
	Corrected value	0.0277	0.0645	0.0632	0.0743	0.0929
Total Recovery (%)		14	32	20	33	38
Corrected Phosphoserine Recovery (mol phosphoserine/mol α-chymotrypsin)		0.072	0.171	0.215	0.116	0.118

Table V. Phosphorus-32 recovery from ^{32}P-DIFP treated cell walls isolated from entire hypocotyls and regions of hypocotyls of etiolated P. vulgaris shown in Figure 1. Excess ^{32}P-DIFP was removed from hypocotyl cell walls by dialysis and from hook and basal cell walls by repeated washings. Values are means of at least triplicate analyses ± S.E.M. Corrected values are the difference between groups pretreated and not pretreated with non-radioactive DIFP. N.S. denotes no significant difference at α=0.01. N.D. denotes value not determined.

Origin of Cell Walls	DIFP pretreatment	No DIFP pretreatment	Corrected value	Protein content	Specific activity
	(pmol ^{32}P mg^{-1} cell wall)			(µg protein/ mg cell wall)	(pmol ^{32}P/ mg cell wall protein)
Entire hypocotyl	7.7 ± 0.1	7.7 ± 0.7	0.0(N.S.)	N.D.	0.0
Hook region	9.1 ± 1.7	14.5 ± 1.8	5.4	128 9	42.2
Basal region	8.9 ± 0.4	12.2 ± 1.9	3.4	110 6	30.9

cell wall, respectively, after removal of excess ^{32}P-DIFP by repeated washing. Recoveries calculated on a cell wall protein basis were 42.2 and 30.9 pmols of ^{32}P mg^{-1} of cell wall protein for hook and basal cell walls respectively.

5. Phosphoserine Recovery from Cell Walls

There were 0.56 pmol of ^{32}P-phosphoserine mg^{-1} of dry cell wall and 4.38 pmol of ^{32}P-phosphoserine mg^{-1} of cell wall protein recovered from high voltage paper electrophoresis of partial hydrolyzates of ^{32}P-DIFP labeled cell walls isolated from hook regions of hypocotyls of etiolated P. vulgaris (Table VI). No significant (t_{4df}=1.49, α=0.01) phosphoserine recovery occurred in ^{32}P-DIFP treated cell walls isolated from basal regions of similar hypocotyls when compared to DIFP-pretreated controls. Total radioactive product recovery from electrophoregrams ranged from 12 to 25%. A mean corrected value of 2.71 pmol of phosphoserine mg^{-1} of cell wall (21.2 pmol of phosphoserine mg^{-1} of cell wall protein) was obtained when phosphoserine recoveries from labeled hypocotyl hook cell walls were corrected for loss during hydrolysis and electrophoresis.

6. Spin Labeling

The spectrum shown in Figure 2a was obtained for HTMFP-labeled α-chymotrypsin in deionized water after removal of excess spin label by either dialysis or TCA precipitation of the protein. The spectrum obtained for HTMFP in phosphate buffer is shown in Figure 2b. No resonance was observed between 3435 and 3535 G at highest gain in intact etiolated P. vulgaris hypocotyls, 1-5 cm segments of the

Table VI. Phosphorus-32-phosphoserine recovered from ^{32}P-DIFP treated cell walls isolated from regions of hypocotyls of etiolated P. vulgaris shown in Figure 1. Values are expressed as means of triplicate analyses S.E.M. Corrected values are the difference between groups pretreated and not pretreated with DIFP. Phosphoserine recoveries were corrected individually for loss during hydrolysis and electrophoresis. N.S. denotes no significant difference at α=0.01. N.D. denotes value not determined.

Origin of Cell Walls	Phosphoserine recovery (pmol ^{32}P-phosphoserine/ mg cell wall)	Corrected phosphoserine recovery (pmol ^{32}P-phosphoserine/ mg cell wall)	Specific phosphoserine recovery (pmol ^{32}P-phosphoserine/ mg cell wall protein)	Corrected specific phosphoserine recovery (pmol ^{32}P-phosphoserine/ mg cell wall protein)
Hook region				
DIFP pretreatment	0.07 ± 0.00	0.34 ± 0.02		
No DIFP pretreatment	0.63 ± 0.14	3.05 ± 0.67		
Corrected value	0.56	2.71	4.38	21.2
Basal region				
DIFP pretreatment	0.27 ± 0.03	1.91 ± 0.16		
No DIFP pretreatment	0.34 ± 0.07	1.79 ± 0.30		
Corrected value	0.07 (N.S.)	-0.12 (N.S.)	N.D.	N.D.

Figure 2. Electron spin resonance spectra of a) α-chymotrypsin labeled with HTMFP, b) HTMFP alone in phosphate buffer, and c) entire hypocotyl cell walls labeled with HTMFP. Spectra were recorded at room temperature at a receiver gain of a) 8×10^4, b) 2.4×10^4, and c) 2×10^4.

Figure 2a

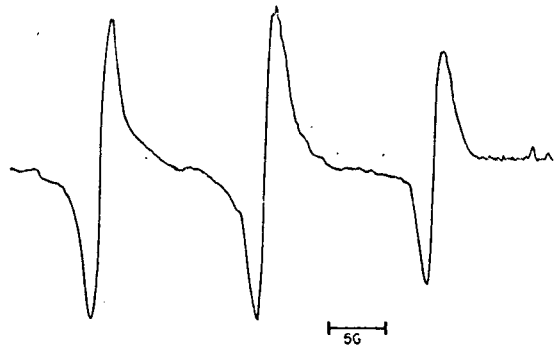


Figure 2b

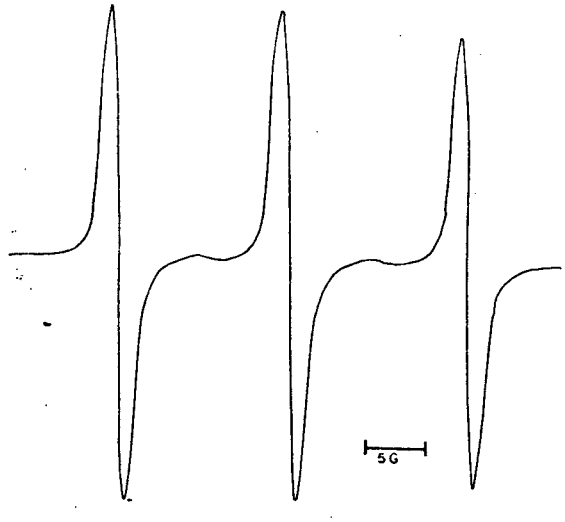
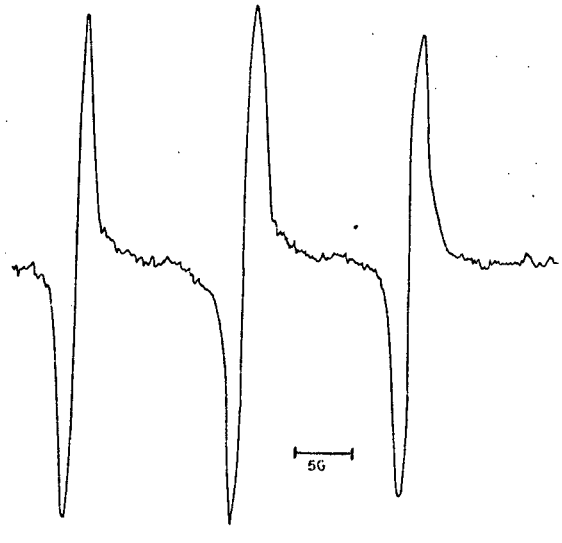


Figure 2c



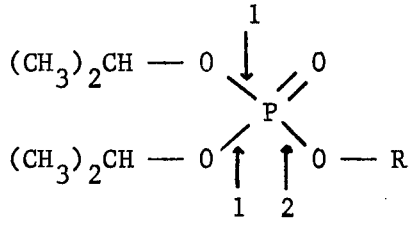
hypocotyls, cell wall preparations, or any of the following HTMFP-treated preparations after removal of excess HTMFP: cellulose powder, DIFP-pretreated cell walls isolated from entire hypocotyls or hook regions of the hypocotyl, and non-pretreated cell walls isolated from the hook region. On one occasion, cell walls isolated from entire hypocotyls were labeled after removal of excess HTMFP by the washing method. This result was not reproducible. The spectrum is shown in Figure 2c. After four days at room temperature, the label was released quantitatively by one wash with deionized water.

D. DISCUSSION

Phosphoserine and orthophosphate were the major radioactive hydrolysis products following the reaction of DIFP with both α-chymotrypsin and P. vulgaris hypocotyl hook cell walls. This observation indicated that serine was the labeled nucleophile in these preparations.

Cohen et al., (1967) stated that "serine itself and its peptides do not react (with DIFP)." The results shown in Table 1 do not support their observation. Phosphoserine was detected in partial hydrolyzates after the reaction of DIFP and serine (Table I). An estimate of the serine which was phosphorylated was difficult to obtain from these values for the following reason. During acid hydrolysis of a DIP ester, all of the bonds indicated in Scheme 1 would be hydrolyzed. The R-phosphate ester would be recovered quantitatively only if the diisopropyl esters 1 were cleaved quantitatively first. Under conditions which yield complete hydrolysis of diisopropylphosphate esters, some hydrolysis of a serine phosphate ester would be expected. The best estimate (a minimal value)

Scheme 1.



would be the maximal value for phosphoserine recovery in Table I (1.1%).

The nearest integer of the mole ratio of ^{32}P-DIP-α-chymotrypsin to α-chymotrypsin active centers was 1.0. This result agreed with observations of Jansen, et al., (1950)and Koshland (1960) and suggested that of all of the serine residues in α-chymotrypsin, only the one in the active center of the enzyme was reactive toward DIFP.

Although the phosphoserine recovered after hydrolysis accounted for less than half of the radioactivity in DIP-α-chymotrypsin, the DIFP labeling strategy was applied to cell walls to establish maximum and minimum values for DIFP binding to serine. Any cell wall serine reactive toward DIFP would most likely be a component of an active center of an enzyme but could represent any serine which was sterically available to DIFP and sufficiently nucleophilic.

The method of removal of excess ^{32}P-DIFP had no effect on the mole fraction of α-chymotrypsin labeled with ^{32}P-DIFP (Table II). This was expected since the spontaneous hydrolysis of the DIP-protein ester procedes very slowly (Cohen, et al., 1967). Greenberg and Nachmansohn (1965) showed that when ^{32}P-DIFP inhibited phosphomonoesterase and no serine was phosphorylated, dialysis resulted in complete release of ^{32}P-DIFP from the enzyme and recovery of enzymatic activity. This was not observed, however, when the protein was precipitated and washed.

There was no significant labeling of entire hypocotyl cell walls when excess reagent was removed by dialysis (Table V). The single cell wall preparation that was spin labeled released the spin label after 4 days. These observations suggested that a cell wall preparation treated with the phosphofluoridate may have been labeled but the label was released with the passage of time, either by spontaneous or phosphatase catalyzed hydrolysis. Although spontaneous hydrolysis occurs, it procedes at a very slow rate. On the other hand, acid phosphatase is a known constituent of cell walls (Lamport and Northcote, 1960; Kivilaan, et al., 1961; Suzuki, 1972) and may have existed in these preparations. The release of the spin label may have resulted from enzymatic reduction of the nitroxide (Smith, 1972).

Although cell walls isolated from both hook and basal regions of the hypocotyl contained radioactivity after removal of excess reagent by repeated washing (Table V), only the hook cell walls contained detectable phosphoserine in the hydrolysis products (Table VI). This peculiarity may be attributed to ^{32}P-DIFP labeling of cell wall components other than serine that were not adequately prelabeled by DIFP, however no other evidence supports the identity of such a site.

The only cell wall preparations which contained any significant reactive serine were those extracted from the hypocotyl hook. The corrected phosphoserine recovery from hypocotyl hook cell walls (2.71 pmol $\cdot$ mg^{-1} cell wall, Table VI) represented a minimum value based on the fact that recovery from α-chymotrypsin was incomplete under all conditions tested; the value in Table V (5.4 pmol $\cdot$ mg^{-1} cell wall) represents a maximum value of reactive serine residues.

To detect spin labeled cell wall suspensions (6 mg ml^{-1}) by electron spin resonance spectrometry, the spin label concentration would have to be approximately 2nnmol mg^{-1} cell wall based on sensitivity calculations described by Bolton, et al., (1972). This is substantially greater than the maximum value for DIFP binding to hook cell wall serine.

The reactive serine in hypocotyl hook cell walls which was absent in basal hypocotyl walls could have been part of the active center of a cell wall enzyme or an enzyme adsorbed to the cell wall, or a part of a structural protein, such as extensin (Lamport, 1965) which was still capable of being glycosylated. Numerous enzymes have been identified in the cell walls of various members of the Dicotyledonae. These include phosphatase (Lamport and Northcote, 1960; Kivilaan, et al., 1961; Suzuki, 1972), pectin methyl esterase (Glasziou, 1959), ascorbic acid oxidase (Newcomb, 1951; Mertz, 1964), invertase (Klis and Akster, 1974; Edelman and Hall, 1964), α- (Murray and Bandurski, 1975) and β-glycosidases (Ashford and McCully, 1970; Klis, et al., 1974; Murray and Bandurski, 1975), ATPase (Kivilaan, et al., 1961), phosphorylase (Kivilaan, et al., 1961) and acetyl cholinesterase (Fluck and Jaffe, 1974; Chapter I of this thesis). Alkaline phosphatase and phosphorylase from animal sources are inactivated by DIFP (Milstein, 1964 and Fisher, et al., 1959, respectively) and it is probable that the plant enzymes are similarly inactivated. Acetylcholinesterase was inactivated by DIFP (Chapter I, Figure 7) and many other esterases are inhibited by DIFP so pectin-methylesterase may be inactivated by DIFP although no direct evidence exists in support of this argument. It

would be possible to account for the labeling of hook cell walls if any of these (or other) DIFP sensitive enzymes had a greater specific activity in the hypocotyl hook than in the basal region of the hypocotyl. This is the case for acetylcholinesterase (Chapter I, Table III). Murray and Bandurski (1975) have identified β-galactosidase in higher specific activity in the hook of pea stems than in the basal stem segments, but DIFP sensitive enzymes have not been studied from this perspective.

Using the value of estimated catalytic center activity of acetylcholinesterase (0.197 units $pmol^{-1}$ DIFP; Chapter II, Table V) and the specific activity of acetylcholinesterase in whole hypocotyls (0.25 units mg^{-1} cell wall protein), the predicted amount of DIFP labeling in the whole hypocotyl cell walls attributable to acetylcholinesterase is 1.25 pmol of DIFP. This quantity represents only 6% of the minimum value for reactive serine (Table VI). However, this value is maximal because the specific activity in hook cell walls may be greater than in entire hypocotyl cell walls (see Chapter I, Table III). This DIFP sensitive enzyme cannot account for all DIFP binding in hook cell walls.

It is also possible that cytoplasmic enzymes adsorbed to the cell wall by ionic interactions (Jansen, et al., 1960) would bind DIFP. Hook cell walls may bind more cytoplasmic enzymes or a greater proportion of DIFP sensitive enzymes may exist in the cytoplasm of hook cells than in that of basal cells. Before resolving these possibilities, the purity of the cell wall preparations must be established.

During the cell wall extraction procedure, the high purity of the preparations was established by the observation that there were fewer than 2% of intact cells and also by failure to find contaminants during examination by phase contrast microscopy. The settling procedure chosen for the separation of intact cells from cell wall fragments, produced good yields of cell wall fragments but some intact cells were present. These were eliminated during the washing procedure, but soluble cell constituents not visible by phase contract microscopy may have been released during the extraction and settling procedures. Adsorbed proteins, for example, may have had a measurable impact on reactive serine since soluble proteases and esterases abound in plant cell extracts.

In any cell wall purification method, one is faced with the problem of retaining native cell wall enzymes yet removing adventitious ones. My procedure was chosen to avoid the use of reagents which would remove ionically bound cell wall enzymes but the risk of contamination by soluble cytoplasmic enzymes was increased.

It is conceivable that hook cell walls would bind more contaminating enzymes than would basal cell walls. Ordin, et al., (1957) observed that methylation of the pectin component of cell walls accompanies auxin-induced cell extension in Avena coleoptiles. If more methyl esters of uronic acids existed in the fully extended cell walls of the basal region, the hook would contain more negatively charged uronic acid carbonyls and provide for ionic interactions with contaminating proteins. No evidence supports the alternative that there are a greater proportion of DIFP sensitive enzymes in the hook than in

the basal region of the hypocotyl.

If the reactive serine observed in the hook cell walls absent from the basal cell walls was glycosylated during cell extension, then the quantity of serine labeled by DIFP should equal the quantity of non-glycosylated serine present in young cell walls which would be glycosylated in situ and exist as glycosyl serine in the fully extended cell wall. Klis (1976) examined the quantity of glycosylated (hydrazine labile) and non-glycosylated (hydrazine stable) serine in segments excised from etiolated pea epicotyl during elongation and after elongation had ceased. He found that glycosylated serine increased from 3.4 to 20.0 nmol mg^{-1} cell wall while non-glycosylated serine increased from 14.6 to 20.9 nmol mg^{-1} cell wall. From these data, if all of the non-glycosylated serine existing in the cell wall became glycosylated during cell extension (which would still not account for all of the glycosylated serine) and if the bean hypocotyl behaved similarly, then at least 14.6 nmol reactive serine would have been expected per mg of hook cell wall. The maximum value (Table V) was actually 3 orders of magnitude less than this. Klis concluded that the glycosylation site is not likely to be in the cell wall because the hydroxyproline: glycosyl-serine ratio remained constant during elongation. The results of this study similarly refute the possibility that the serine glycosylation site is in the cell wall.

It may be possible to use the radioactive DIFP labeling strategy as a rapid method to detect ionically bound proteins, whether native or adventitious, and thereby establish a criterion of purity, if cell walls which have been purified by washing with 1 M-NaCl, 8 M-urea, 1 M-NH_4OH

or 0.5 M-formic acid (Mitchell and Taylor, 1969) can first be demonstrated to contain no reactive serine. Such a strategy would use the labeling procedure described in Methods and the washing procedure to remove excess ^{32}P-DIFP (or any other radioactive isotope of DIFP). The difference between the counts per minute of DIFP pretreated and non-pretreated cell walls would be a direct measure of purity.

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CHAPTER II

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