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Cholinesterases from Plant Tissues

III. DISTRIBUTION AND SUBCELLULAR LOCALIZATION IN *PHASEOLUS AUREUS* ROXB.[1]

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ABSTRACT

The distribution and localization of cholinesterase in *Phaseolus aureus*, *Glycine max*, and *Pisum sativum* is described. The enzyme is present in roots, leaves, stems, root callus tissue, root cells suspension cultures, and root nodules. Cholinesterase in roots is found primarily in the cell wall. In cell fractionation experiments, at least 95% of the cholinesterase activity is associated with cell wall material. The enzyme can be solubilized by salt solutions, whereas Triton X-100 and sodium deoxycholate solubilize relatively small amounts of the enzyme. Cytochemical techniques have been employed to show the presence of cholinesterase activity at the cell surface and in the cell wall of certain cells of the root.

Acetylcholine has been reported to be present in extracts prepared from leaves, stems, buds, and roots of *Phaseolus aureus* Roxb., the mung bean (18, 19). More recently a cholinesterase has been extracted and purified from *P. aureus* tissue (31, 32). In studying the functional aspects of ACh[2] biochemistry in plants, it is necessary to ascertain its location in the organs, tissues, and cells of the plant. Although ACh cannot be localized using cytochemical techniques, there are several methods available which can be used to localize ChE activity. This report will present biochemical, cytochemical, and cell fractionation data which describe the distribution and localization of ChE in *P. aureus*.

MATERIALS AND METHODS

Materials. Seeds of *Phaseolus aureus* Roxb. and *Pisum sativum* L. var. Alaska were soaked in aerated tap water for 24 hr and grown in vermiculite in growth chambers (16-hr photoperiod) at 24 C. Dark-grown plants were harvested under a green safelight. Root callus tissue, root cell suspension cultures, and root nodules (all derived from *Glycine max* L. Merr.) were provided by Dr. M. Reporter of the C. F. Kettering Laboratory, Yellow Springs, Ohio.

The chemicals were purchased from the following sources: acetylthiocholine chloride, DTNB, neostigmine bromide, Triton X-100, sodium deoxycholate, and choline chloride (Sigma Chemical Co., St. Louis, Mo.); glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.); sucrose, special enzyme grade (Schwarz Mann, Orangeburg, N.Y.); and ammonium sulfate (Mallinckrodt Chemical Works, St. Louis, Mo.). The buffer used was 10 mM K phosphate, pH 7.0, unless otherwise specified.

Most of the subcellular fractions were prepared using the HB-4 rotor in a Sorvall RC-2B centrifuge. A TY-65 fixed-angle rotor and a Beckman L2-65B ultracentrifuge were used to obtain the 100,000*g* pellet and supernatant fraction.

Extraction of Cholinesterase. The method of extraction is that described by Riov and Jaffe (32) and was carried out at 4 C. Tissues from seedlings were homogenized in two volumes (v/w) of buffer in a VirTis homogenizer, and the homogenate was stirred for 30 min and was filtered through two layers of a 54-μm mesh nylon net. The enzyme in the residue was solubilized by extracting the material collected from the nylon net with two volumes of 4% (w/v) $(NH_4)_2SO_4$ in buffer. After regrinding the residue, the homogenate was stirred for 60 min, filtered through nylon net, and centrifuged for 15 min at 20,000*g*. The enzyme solubilized in 4% (w/v) $(NH_4)_2SO_4$ was precipitated from the solution by adding solid $(NH_4)_2SO_4$ up to 80% saturation and redissolving the resulting precipitate in buffer. This solution was dialyzed against buffer overnight, and the dialysate was centrifuged to clarify the extract.

Cholinesterase Assays. ChE activity was measured photometrically using a modification of the method of Ellman *et al.* (9). One of two procedures was followed depending on whether we were assaying extracted and solubilized ChE or assaying particulate fractions. In the case of extracted and solubilized enzyme, the standard reaction mixture in a final volume of 0.54 ml contained 0.25 ml of 0.5 M K phosphate, pH 8.0; 20 μl of 2.6 mM DTNB prepared in buffer containing 37.5 mg $NaHCO_3$/100 ml, and 0.25 ml of enzyme. A control assay using preincubation with neostigmine bromide at a final concentration of 25 μM was also performed to correct for the spontaneous hydrolysis of acetylthiocholine, for the reaction of DTNB with free sulfhydryl groups in the preparation, and for the hydrolysis of acetylthiocholine by other enzymes present in the preparation. Neostigmine has been identified as a potent and specific inhibitor of ChE activity in these preparations (32). After preincubation at 37 C for 30 min, the reaction was initiated by the addition of 20 μl of 12.5 mM acetylthiocholine chloride. The mixtures were incubated for 12 min and the absorbance determined at 412 nm. Activity was calculated as nanomoles of acetylthiocholine hydrolyzed per min, based on 1.36×10^4 for the molar extinction coefficient of the yellow anion (2-nitro-5-thiobenzoate) formed during the reaction.

To assay particulate fractions the standard reaction mixture volume (and that of all ingredients) was increased 2.5-fold.

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[2] Abbreviations: ACh: acetylcholine; ChE: cholinesterase; DTNB: 5,5-dithiobis-(2-nitrobenzoic acid).

Following the incubation period, the reaction mixture was immediately filtered with suction through one Whatman GF/B glass fiber filter. The filtrate was collected in a cooled centrifuge tube, centrifuged at 20,000*g* for 10 min and the absorbance of the supernatant fluid was determined. In both assay procedures the concentration of ChE was adjusted so that the net absorbance was directly proportional to the amount of enzyme assayed (32).

ChE activity was also assayed in intact roots from *P. aureus*. Individual roots systems were cut from the plant and placed in 20.25 ml of the assay medium at 37 C. Aliquots of 0.5 ml were removed at regular intervals and the A_{412} determined. At 4 min, neostigmine bromide (to give a final concentration of 25 μM) was added to the medium.

Riov and Jaffe (32) reported that $(NH_4)_2SO_4$ catalyzes the hydrolysis of acetylthiocholine. We have found that sucrose also acts as a catalyst, interfering with the ChE assay and giving high absorbance values in control assays. Therefore fractions containing high concentrations of either $(NH_4)_2SO_4$ or sucrose were dialyzed against buffer before they were assayed.

Cell Fractionation. Roots of 12-day-old seedlings of *P. aureus* were homogenized with a mortar and pestle with acid-washed sand in two volumes (v/w) of homogenizing medium (0.44 M sucrose; 0.05 M K phosphate, pH 7.4). The crude homogenate was filtered through two layers of nylon net and both fractions were saved. The filtrate was fractionated using either differential centrifugation or sucrose discontinuous gradient centrifugation. In the first case, the homogenate was first centrifuged at 1,000*g* for 10 min. The supernatant fluid was collected and the pellet was resuspended, dispersed in a small volume of the homogenizing medium, and recentrifuged. The supernatant fluid from the wash was combined with the first supernatant fluid, the combined supernatant fluids were centrifuged at 10,000*g* for 10 min, and the pellet was washed once with homogenizing medium. The 10,000*g* supernatant fluid and wash were centrifuged at 100,000*g* for 90 min. The three pellets were individually suspended in buffer and dialyzed against buffer overnight. An aliquot of the 100,000*g* supernatant fluid was also dialyzed. In one experiment, $(NH_4)_2SO_4$ or Triton X-100 was added to the filtrate to give final concentrations of 4% (w/v) and 0.5% (v/v), respectively. The residual plant material was resuspended in buffer and homogenized in a VirTis homogenizer. An aliquot of this homogenate was also dialyzed.

A one-step sucrose discontinuous density gradient was prepared by first adding 5.0 ml of 2 M sucrose (containing 0.05 M K phosphate, pH 7.4) to a 30-ml centrifuge tube. Twenty-five ml of homogenate filtrate were layered over this step, and the preparation was centrifuged at 27,000*g* for 5 hr. Three fractions were collected from the tube: the supernatant fluid, the material which accumulated at the interface of the supernatant fluid and the sucrose step (interface fraction), and a pellet. The pellet was resuspended in buffer and the three fractions were dialyzed against buffer overnight. In one experiment, $(NH_4)_2SO_4$ or Triton X-100 was added to the filtrate and sucrose step to give a final concentration of 4% (w/v) and 0.5% (v/v), respectively. In another experiment the interface fraction was divided into four equal aliquots and diluted with 0.05 M K phosphate, pH 7.4. Additions were made to each of the aliquots to give the following compositions: (*a*) no addition (control); (*b*) 4% (w/v) $(NH_4)_2SO_4$; (*c*) 0.5% (v/v) Triton X-100; or (*d*) 0.2% (w/v) sodium deoxycholate. The samples were then centrifuged at 100,000*g* for 90 min, the pellets resuspended in buffer, and the resuspended pellets and supernatant fluids dialyzed against buffer overnight.

Solubilization Studies. Tissues from *P. aureus* or *Pisum sativum* were homogenized in 10 volumes of buffer in a VirTis homogenizer and centrifuged at 27,000*g* for 20 min. The supernatant fluid, which contained less than 2% of the total ChE activity, was discarded and the pellet was resuspended in the medium to be tested using approximately the equivalent of 5 ml/g fresh weight. The suspension was vigorously stirred for 1 hr and centrifuged at 27,000*g* for 20 min. The pellet was suspended in buffer (using the equivalent of 5 ml/g fresh weight) and both the suspension and supernatant fluid were dialyzed against buffer overnight.

Cytochemical Studies. The root systems from 12-day-old seedlings of *P. aureus* were fixed for 1 hr in 2% (v/v) glutaraldehyde in 0.1 M K phosphate, pH 7.0, at 4 C. They were then rinsed for 1 hr with several changes of 0.1 M K acetate, pH 6.0, or in the acetate buffer containing 0.5 mM neostigmine bromide. Following the rinse period the roots were placed in Karnovsky-Roots cytochemical medium (21) prepared in the acetate buffer and using acetylthiocholine chloride as substrate. Following an appropriate period of incubation at room temperature, the roots were rinsed for 1 hr with several changes of distilled water. Four separate controls were used: (*a*) the incubation in the Karnovsky-Roots medium was omitted; (*b*) neostigmine bromide was added to the medium to a final concentration of 0.5 mM; (*c*) the substrate was omitted from the medium; or (*d*) the root systems, following fixation and rinsing, were suspended over a boiling water bath for 30 min.

Two alternative method were employed to prepare the roots for examination. Roots which were to be examined with the light microscope were dehydrated through an ethanol series and finally placed in glycerol. Roots which were to be examined by electron microscopy were post fixed with osmium tetroxide following the water wash. They were then dehydrated with ethanol, washed with propylene oxide, and embedded in Araldite-Epon. Silver and gold sections were cut using glass knives in a Reichert OM U2 ultramicrotome and were examined with a Siemens Elmiskop I electron microscope.

RESULTS

Cholinesterase Activity in Plant Organs. ChE is present in all of the organs of *P. aureus* that were examined from both light- and dark-grown plants (Table I). The values in this table represent extracted and purified enzyme activity and therefore are only a portion of the total enzyme activity initially present in the tissues. We have also identified ChE activity in callus tissue, root cell suspension cultures, and root nodules, all derived from *Glycine max*.

Cell Fractionation. The data from the differential centrifugation and sucrose density gradient centrifugation fractionations (Tables II and III) indicate that from 90 to 95% of the total ChE activity is present in the plant residue material trapped on the nylon net. ChE activity in the filtrate is associated with particulate matter and is heterodisperse, with ChE present in all the pellets and absent from the 100,000*g* super-

Table I. *Cholinesterase Activity Extracted from Plant Parts of Phaseolus aureus*

Plant Part	Extractable Cholinesterase Activity	
	Dark-grown	Light-grown
	nmoles/min·g fresh wt	
Bud		0.4
Leaves	1.5	1.1
Hypocotyls or stems	0.06	0.7
Roots	1.6	2.2

Table II. *Distribution of Cholinesterase Activity in Subcellular Fractions Prepared by Differential Centrifugation*

Fraction	Cholinesterase Activity		
	Control	0.5% (v/v) Triton X-100	4% (w/v) $(NH_4)_2SO_4$
	%		
Residue	94.3 ± 2.3[1]	96.6	92.4
Filtrate			
1,000*g* Pellet	1.6 ± 0.5	0.9	0.4
10,000*g* Pellet	1.0 ± 0.4	1.1	0.1
100,000*g* Pellet	3.1 ± 0.9	0.4	0.2
Supernatant fluid	0	0.9	6.8

[1] SD.

Table III. *Distribution of Cholinesterase Activity in Subcellular Fractions Prepared by Sucrose Discontinuous Density Gradient Centrifugation*

Fraction	Cholinesterase Activity		
	Control	0.5% (v/v) Triton X-100	4% (w/v) $(NH_4)_2SO_4$
	%		
Residue	91.9 ± 3.4[1]	88.6	86.5
Filtrate			
Supernatant fluid	0.9 ± 0.2	2.4	10.8
M fraction	2.3 ± 1.0	3.0	2.5
Pellet	4.9 ± 1.3	6.0	0.2

[1] SD

Table IV. *Solubilization of Cholinesterase from Phaseolus aureus Roots by Media*

Medium	Cholinesterase Activity Solubilized
	%
(1) 0.01 M K phosphate, pH 7.0	0.8
(2) 0.5% (v/v) Triton X-100 in No. 1	5.9
(3) 0.2% (w/v) Na deoxycholate in No. 1	3.1
(4) 0.1 M EDTA in No. 1	7.5
(5) 0.2 M K borate, pH 8.5	6.2
(6) 0.2 M $CaCl_2$	2.2
(7) 0.6 M choline chloride in No. 1	4.6
(8) 0.6 M KCl in No. 1	29.0
(9) 4% (w/v) $(NH_4)_2SO_4$ in No. 1	36.7

natant fluid (Table II). When Triton X-100 or $(NH_4)_2SO_4$ is added to the filtrate, the distribution of ChE activity among the four fractions is altered, particularly in the latter case. There is a loss of activity from the three pellets and an increase in the ChE activity in the supernatant fluid. Approximately 60% of the ChE activity in the filtrate passes through 2 M sucrose (Table III). Lesser amounts are found in the supernatant fluid and in the interface fraction. $(NH_4)_2SO_4$ (4% [w/v]) in the filtrate and sucrose step causes large changes in the distribution of ChE activity among the three fractions, but 0.5% (v/v) Triton X-100 does not effect such changes. The most striking change is the removal of nearly all ChE activity from the pellet and the activity's appearance in the supernatant fluid. When the solubility properties of the ChE in the interface fraction were examined we found no ChE was solubilized in the control or Triton X-100 treatments; however 4% (w/v) $(NH_4)_2SO_4$ solubilized nearly half the ChE and 0.2% (w/v) deoxycholate solubilized approximately 20%.

Solubilization Studies. Various media were tested for their ability to solubilize ChE activity from root tissue of 12-day-old *P. aureus* seedlings (Table IV). Of the nine media tested only 0.6 M KCl and 4% (w/v) $(NH_4)_2SO_4$ solubilized a substantial portion of ChE. We observed no loss of ChE activity during any of these treatments.

In a second experiment, particulate material prepared from *P. aureus* seedlings of different ages was extracted with 4% (w/v) $(NH_4)_2SO_4$ in buffer. On a fresh weight basis, the total ChE activity doubles from day 6 to day 18 (Table V). However, the fraction of ChE activity which can be solubilized with 4% (w/v) $(NH_4)_2SO_4$ decreases from 50% in 6-day-old roots to about 20% in 18-day-old roots. Thus the increase in total activity is due to an increase in the insoluble fraction of ChE.

In a third study, several tissues from 12-day-old seedlings of *P. aureus* and *P. sativum* were examined for total and soluble ChE (Table VI). On a fresh weight basis, the roots have the highest amount of ChE, and both the total and the soluble ChE vary with the tissue. Two internodes from light-grown pea stems were examined: No. 3 internode, an internode which had ceased elongating; and No. 5 internode, an internode which was still elongating. The total ChE activity as well as the fraction of the activity soluble in 4% (w/v) $(NH_4)_2SO_4$ in the third internode is about equal to that in the fifth internode.

Cholinesterase Assays of Intact Roots. ChE activity can be detected at the surface of the roots of *P. aureus* (Fig. 1). The A_{412} of the assay medium begins to increase immediately after the addition of the substrate. Furthermore, the addition of neostigmine to the medium results in complete inhibition of the ChE activity within 4 min. The rate of substrate hydrolysis observed in the presence of 25 μM neostigmine bromide is equal to the rate of nonenzymatic hydrolysis at pH 8.0.

Table V. *Total and $(NH_4)_2SO_4$-Soluble Cholinesterase Activity in Roots from Phaseolus aureus of Different Ages*

Age of Plants	Total	Insoluble	Insoluble
days	*nmoles/min·g fresh wt*		%
6	34.7	17.1	49.3
8	32.5	15.7	48.3
10	40.4	28.1	69.5
12	42.5	29.8	70.2
14	69.2	48.4	70.0
16	68.2	50.2	73.6
18	69.7	54.3	77.9

Table VI. *Total and $(NH_4)_2SO_4$-Soluble Cholinesterase Activity from Various Organs*

Plant Part	Total Activity	Soluble Activity
	nmoles/min·g fresh wt	*% of total*
Buds[1]	17.1	40.4
Leaves[1]	5.35	37.4
Hypocotyl[1]	2.00	56.8
Roots[1]	42.5	29.8
Internode No. 3[2]	6.55	73.1
Internode No. 5[2]	6.60	67.6

[1] 12-day-old light-grown *Phaseolus aureus*.
[2] 12-day-old light-grown *Pisum sativum*.

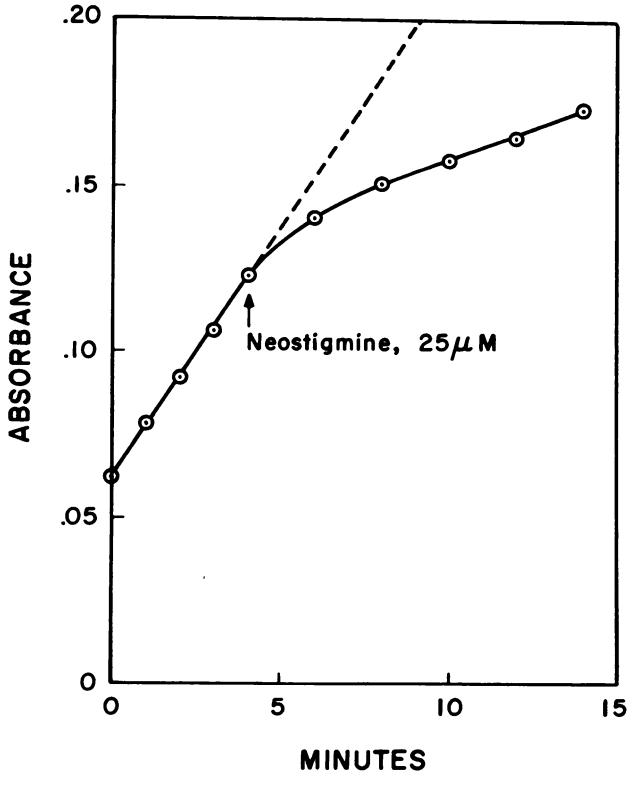

FIG. 1. ChE activity assayed with intact roots. A root system from a 7-day-old dark-grown *P. aureus* seedling was placed in the assay medium and preincubated at 37 C for 5 min. At T = 0 acetylthiocholine was added and at T = 4 min neostigmine bromide was added to a final concentration of 25 μM.

Cytochemical Studies. The final reaction product of the cytochemical reaction is copper ferrocyanide, a reddish brown, electron-dense precipitate. Thus the same preparations can be used for both light and electron microscopic studies. When the roots are incubated in the Karnovsky-Roots medium a visible reddening of the roots occurs. When control preparations are compared to the experimental preparation a difference in intensity of red color is apparent after a 30 min incubation period at room temperature; and the difference becomes more pronounced during incubation periods of 2 to 3 hr. The difference is apparent to the unaided eye throughout the length of the root system and this observation was verified with the use of a microspectrophotometer (data not shown).

Roots which were processed for electron microscopic observations were incubated for 40 min or less. If longer incubation periods were used there was a high density of background reaction product present, most likely a result of the autohydrolysis of acetylthiocholine.

We have examined two regions of the roots of *P. aureus*. Most observations have been of sections made from approximately 500 μm behind the tip where there are still one or two layers of root cap cells present (Fig. 2A). Other sections have been prepared from a region behind the root cap where the epidermal cells have begun to vacuolate. Even after the short incubation periods employed, there is considerable background reaction product present. This background material is particularly evident in root cap cell cytoplasm and in the cell walls between adjacent root cap cells (Fig. 2B). Occasionally the electron-dense material can be seen over the cytoplasm of other cells (Fig. 2E), but it is neither consistent from one cell to another nor associated with any cytoplasmic organelle in particular. The only sites where we have consistently observed reaction product in the experimental materials and not in the control treatments is in the cell walls between preepidermal, epidermal, and cortical cells (Fig. 2, E-H). In these walls, the reaction product can be seen immediately adjacent to the plasma membrane (Fig. 2, E and H) and also in the interior of the cell wall (Fig. 2, E, F, and H). In cells in which the protoplast has pulled away from the cell wall, the reaction product can be seen to remain alongside the cell wall (Fig. 2G).

DISCUSSION

Two components of acetylcholine metabolism have been identified in *Phaseolus aureus:* ACh (18, 19) and a cholinesterase (31, 32). Both molecules have been shown to be present in all the organs of both light- and dark-grown plants, and both are found in highest amounts in the roots and in the aerial buds (18, 19, 31, 32). These components are not present in equal amounts, on a fresh weight basis, in all the plant organs examined and the functional significance of these differences is not understood at this time.

The primary finding of the work reported here is the localization of ChE in the cell wall. First, the cell fractionation studies indicate that at least 95% of the ChE in roots is associated with cell walls. From 90 to 95% of the ChE remains with the plant residue material on the nylon net and over half of the ChE in the filtrate can sediment through 2 M sucrose, a property which indicates that the enzyme is probably associated with cell wall material (23). The solubility properties of the enzyme also point to an association between ChE and cell walls. Triton X-100 (4, 7, 8, 15, 16, 26, 27) and sodium deoxycholate (4, 7, 8), agents which are often used to solubilize membrane-associated proteins, solubilize relatively little ChE in our preparations (Table IV). Furthermore, 0.5% (v/v) Triton X-100 was relatively ineffective in solubilizing ChE from the various subcellular fractions prepared from *P. aureus* (Tables II and III). However, salt solutions which are often used to elute enzymes from cell walls (24) solubilize up to 50% of the ChE in our preparations (Table V). Ammonium sulfate (4%, w/v) also solubilized a substantial portion of the ChE from the subcellular fractions (Tables II and III). However, it should be noted that reagents such as 0.1 M EDTA (10) and 0.2 M $CaCl_2$ (5), which have been used by others to solubilize protein components from cell walls, were relatively ineffective in solubilizing ChE. The results of the assays with intact roots also indicate that ChE is present at the cell surface (2, 3, 25).

In spite of these data supporting an association between ChE and the cell wall, there are many reports in the literature documenting the spurious binding of solubilized enzymes to cell walls. For example, Jansen *et al.* (20) have shown that large amounts of exogenous pectin esterase, pepsin, peroxidase, and α-chymotrypsin will bind to washed cell wall preparations from *Avena* coleoptiles. Hawker (14), in a study of invertase activity in grapes, suggested that tannins may be important in the formation of artifactual protein-cell wall associations, but he was able to solubilize invertase with borate or Triton X-100. Arnold (1) has reported the solubilization of a β-fructofuranosidase from grape berries with 0.2 M borate, pH 8.5. In our system, 0.2 M borate, pH 8.5, solubilized relatively little ChE. Some authors (12, 23, 27, 29) have suggested that some of the enzymes associated with cell wall preparations are constituents of plasma membrane fragments adhering to the walls and to membranes in plasmadesmata. However, in the case of monovalent ion-stimulated ATPase, an enzyme which has been demonstrated to be present in purified plasma membrane preparations (17), the amount of the enzyme which is found

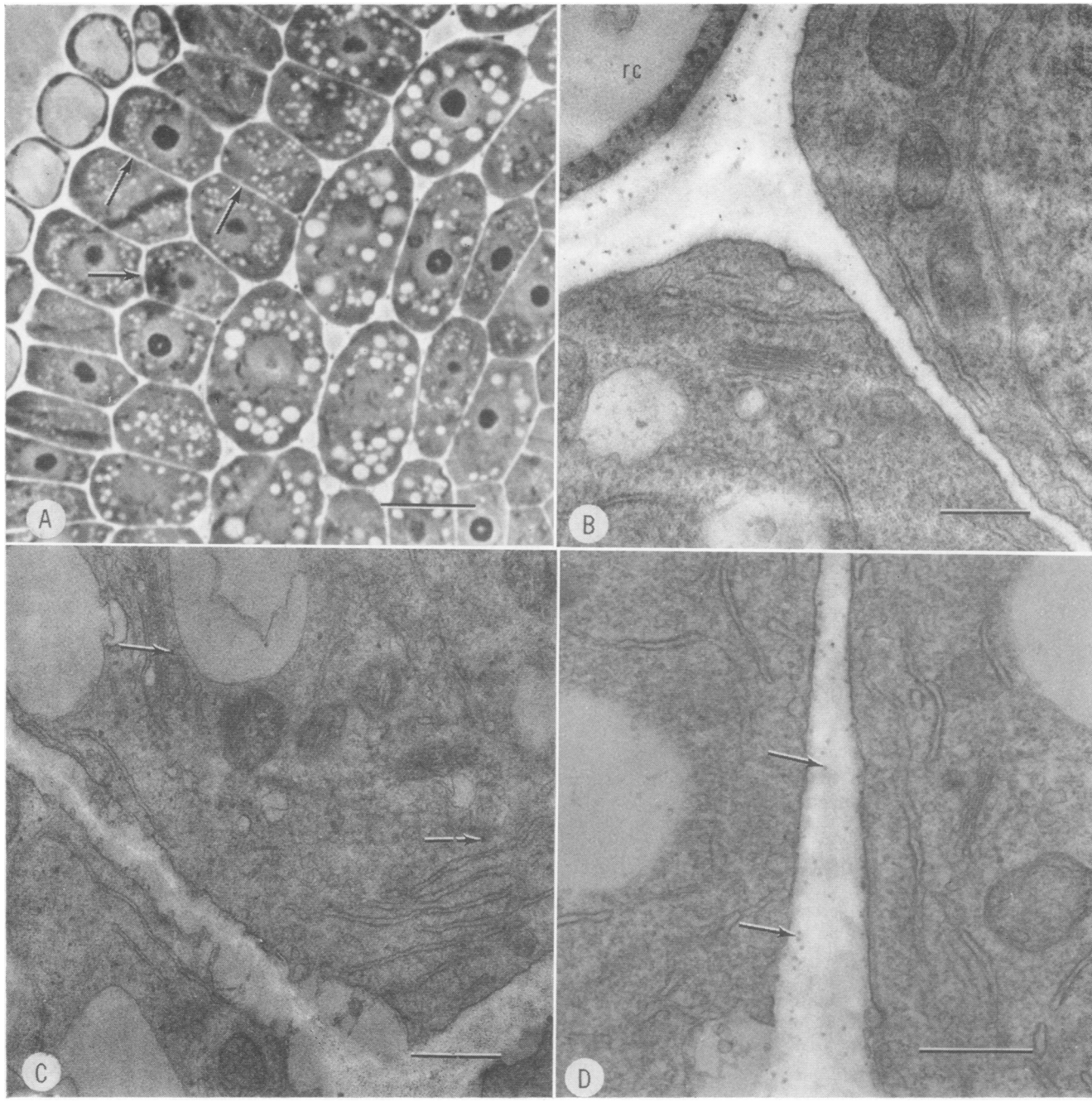

FIG. 2. Cytochemical localization of ChE in *P. aureus* roots. The roots from which these micrographs were made were incubated in the cytochemical medium for 40 min at room temperature. A–D: control treatments. A: phase-contrast micrograph showing the general region of the root on which most of our observations were made. Arrows indicate cell walls in which we have identified ChE activity; bar = 20μm. B: minus substrate control. Grains are present in the root cap cell cytoplasm (rc) but nearly absent from the cell walls; bar = 0.5μm. C: heat-inactivated control. Electron dense material is scattered throughout the cytoplasm of these two pre-epidermal cells but there is little or no reaction product in the cell walls; bar = 0.5μm. D: plus neostigmine control. A few scattered electron-dense grains can be seen in cell wall between these two epidermal cells; bar = 0.5μm. E-H: experimental treatment. E: portions of two pre-epidermal cells are shown here. The reaction product can be seen at the interface of the plasma membrane and the cell wall (arrow) and in the interior of the wall (double arrows). A few scattered grains can be seen in the cytoplasm; bar = 0.9μm. F: portions of two epidermal cells showing the reaction product in the cell wall interior (double arrows) and at the interface of the cell wall and the plasma membrane (arrow); bar 0.9μm. G: in cells in which the plasma membrane has pulled away from the cell wall the reaction product is found with the cell wall; bar = 0.9μm. H: Portions of two pre-epidermal cells (PE) and one cortical cell (CO) can be seen. The reaction product here is seen primarily at the interface of the plasma membrane and cell wall (arrows) but some is present in the interior portion of the wall also (double arrow); bar = 0.9μm.

in the wall fraction is considerably less than we report here (11).

The cytochemical evidence presented here gives independent verification of the cell wall localization of ChE. We have identified ChE activity in radial and tangential cell walls between pre-epidermal, epidermal, and cortical cells. The enzyme appears to be present in the interface between the protoplast and the cell wall, and also in the interior of the wall. We

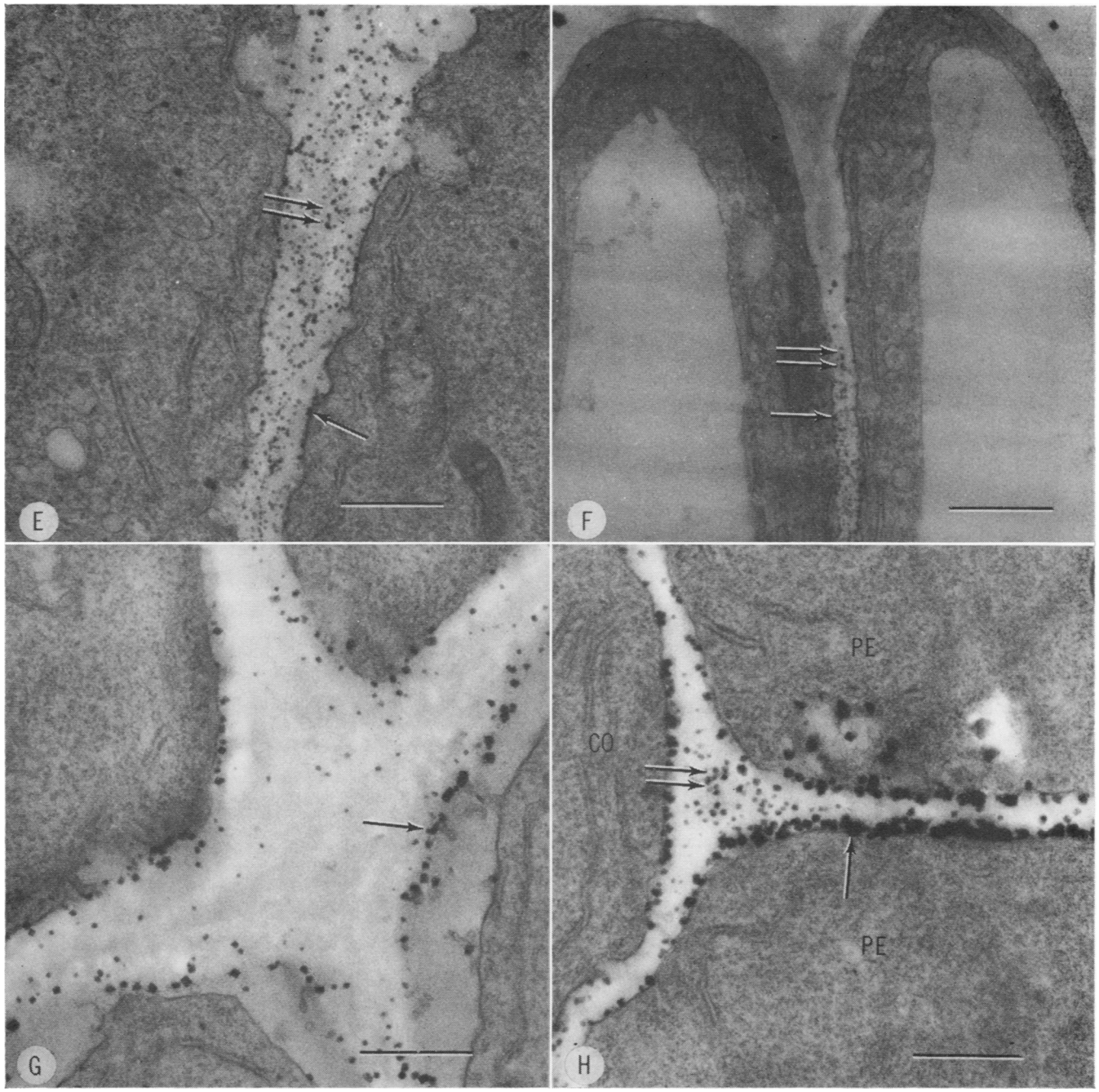

FIG. 2

have neither observed a polarization of the activity nor have we observed an association between ChE and plasmadesmata. It is possible that ChE is a part of the plasma membrane and that the reaction product of the cytochemical reaction diffuses into the cell wall where it forms the accumulations observed in the micrographs. However, Hall (12), in a report of a cytochemical study of ATPase activity in maize roots, presented micrographs showing restriction of the final reaction product to the interface between the protoplast and the cell wall and over plasmadesmata. Since we do not see this kind of picture, we conclude that the reaction product present in the interior of the cell wall represents sites of ChE activity in that region of the wall. The histochemical studies are not exhaustive because we have examined only a relatively small portion of the cells present in the root. However, we have never observed ChE activity in the cytoplasm of any of the cells which we have examined, and there is no evidence from the cell fractionation studies to indicate that a large proportion of the enzyme is associated with cytoplasmic organelles. We conclude that ChE is a native constituent of the cell wall in the cells that were examined.

However, a small amount of ChE appears to be associated with cytoplasmic organelles. For example, from 2 to 3% of the total ChE in the roots is found in the interface fraction, which probably contains mitochondria, tonoplast membranes, plasma membranes, disrupted Golgi bodies, and microsomal material. The ChE activity in this fraction may be a native constituent of some of these membranes or it may be present as an artifact. This activity could also represent nascent wall material present in cytoplasmic vesicles. At least two possible explana-

tions can be given to account for the absence of any cytochemical evidence for intracellular ChE. First, the components of the cytochemical medium may not penetrate the plasma membrane. However, we have observed electron-dense reaction product material in the cytoplasm of cells in both control and experimental treatments. The reaction product does not appear to be associated with any subcellular organelle in particular. Second, we may speculate that ChE is found intracellulary in large amounts only in immature cells which have not yet been examined cytochemically. We cannot distinguish between these possibilities with the information we have now but this question is under investigation.

The wall-associated ChE appears to be present in two forms —one readily solubilized by 4% (w/v) $(NH_4)_2SO_4$ and another which is insoluble under these same conditions. This phenomenon has been observed with at least five other cell wall-associated enzymes (6, 13, 22, 28, 30), and in one case has been suggested to be due to the presence of noncovalently and covalently bound populations of the enzyme (30). We have no evidence to indicate a covalent attachment between ChE and components of the cell wall. Riov and Jaffe (32) reported that the ChE could be extracted from *P. aureus* roots in two different molecular weight forms—one with an estimated mol wt of 80,000, and another with a mol wt greater than 2,000,000. We do not know if there is any relationship between these two molecular forms and the two populations which differ in their solubility in 4% (w/v) $(NH_4)_2SO_4$.

Finally, the information we have reported here may be useful in answering questions of the function of these molecules in plants. The information we now have regarding the distribution of ACh and ChE activity throughout the plant indicates that any hypothesis of the action of ACh in plants must take this into account. The cell wall localization of ChE may indicate one of several functions. The enzyme may be part of a rapid intercellular communication system between cells. However, such a system seems superfluous in the presence of plasmadesmata. The hypothesis proposed by Jaffe (19) and subsequently supported by more recent data (31) is that ACh is a native metabolic regulator in *P. aureus*. Our data regarding the cell wall localization of ChE are not inconsistent with this hypothesis. However, more information is needed to confirm this hypothesis.

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LITERATURE CITED

1. Arnold, W. N. 1966. β-Fructofuranosidase from grape berries. II. Solubilization of a bound fraction. Biochim. Biophys. Acta 128: 124–129.
2. Brevin, N. J. and D. H. Northcote. 1973. Partial purification of a cyclic AMP phosphodiesterase from soybean callus. Isolation of a nondialysable inhibitor. Biochim. Biophys. Acta 320: 104–122.
3. Chang, C. W. and R. S. Bandurski. 1964. Exocellular enzymes of corn roots. Plant Physiol. 39: 60–64.
4. Chiu, T. H., J. O. Dolly, and E. A. Barnard. 1973. Solubilization from skeletal muscle of two components that specifically bind α-bungarotoxin. Biochem. Biophys. Res. Commun. 51: 205–213.
5. Chrispeels, M. J. 1969. Synthesis and secretion of hydroxyproline containing macromolecules in carrots. I. Kinetic analysis. Plant Physiol. 44: 1187–1193.
6. Copping, L. D. and H. E. Street. 1972. Properties of the invertases of cultured sycamore cells and changes in their activity during culture growth. Physiol. Plant 26: 346–354.
7. Cuatrecasas, P. 1972. Isolation of the insulin receptor of liver and fat-cell membranes. Proc. Nat. Acad. Sci. U.S.A. 69: 318–322.
8. Eldefrawi, M. E., A. T. Eldefrawi, S. Seifert, and R. D. O'Brien. 1972. Properties of Lubrol solubilized acetylcholine receptor from *Torpedo* electroplax. Arch. Biochem. Biophys. 150: 210–218.
9. Ellman, G. L., K. D. Courtney, V. Andres, Jr., and R. M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholine esterase activity. Biochem. Pharmacol. 7: 88–95.
10. Fan, D. F. and G. A. Maclachlan. 1967. Studies on the regulation of cellulase activity and growth in excised pea epicotyl sections. Can. J. Bot. 45: 1837–1844.
11. Fisher, J. and T. K. Hodges. 1969. Monovalent ion-stimulated adenosine triphosphatase from oat roots. Plant Physiol. 44: 385–395.
12. Hall, J. L. 1969. Localization of cell surface adenosine triphosphatase activity in maize roots. Planta 85: 105–107.
13. Hall, J. L. and V. S. Butt. 1968. Localization and kinetic properties of β-glycerophosphatase in barley roots. J. Exp. Bot. 19: 276–287.
14. Hawker, J. S. 1969. Insoluble invertase from grapes; an artifact of extraction. Phytochemistry 8: 337–344.
15. Heller, J. S. and C. L. Villemez. 1972. Solubilization of a mannose-polymerizing enzyme from *Phaseolus aureus*. Biochem. J. 128: 243–252.
16. Henderson, R. and J. H. Wang. 1972. Solubilization of a specific tetrodotoxin-binding component from garfish olfactory nerve membrane. Biochemistry 11: 4565–4569.
17. Hodges, T. K., R. T. Leonard, C. E. Bracker, and T. W. Keenan. 1972. Purification of an ion-stimulated ATPase from plant roots: association with plasma membranes. Proc. Nat. Acad. Sci. U.S.A. 69: 3307–3311.
18. Jaffe, M. J. 1970. Evidence for the regulation of phytochrome-mediated processes in bean roots by the neurohumor, acetylcholine. Plant Physiol. 46: 768–777.
19. Jaffe, M. J. 1972. Acetylcholine as a native metabolic regulator of phytochrome-mediated processes in bean roots. *In:* V. C. Runeckles and T. C. Tso, eds., Recent Advances in Phytochemistry, Vol. 5. Academic Press, New York. pp. 81–104.
20. Jansen, E. F., R. Jang, and J. Bonner. 1960. Binding of enzymes to *Avena* coleoptile cell walls. Plant Physiol. 35: 567–574.
21. Karnovsky, M. J. and L. Roots. 1964. A "direct-coloring" thiocholine method for cholinesterases. J. Histochem. Cytochem. 12: 219–221.
22. Klis, F. M. 1971. α-Glucosidase activity located at the cell surface in callus of *Convolvulus arvensis*. Physiol. Plant 25: 253–257.
23. Lai, Y. F. and J. E. Thompson. 1970. 5-Nucleotidase and glucose-6-phosphatase in a purified cell-wall fraction from *Phaseolus vulgaris*. Phytochemistry 9: 1017–1021.
24. Lamport, D. T. A. 1965. The protein component of primary cell walls. Advan. Bot. Res. 2: 151–218.
25. Mandels, G. R. 1953. The properties and surface location of an enzyme oxidizing ascorbic acid in fungus spores. Arch. Biochem. Biophys. 42: 164–173.
26. McFarland, B. H. and G. Inesi. 1971. Solubilization of sarcoplasmic reticulum with Triton X-100. Arch. Biochem. Biophys. 145: 456–464.
27. Miledi, R., P. Molinoff, and L. T. Potter. 1971. Isolation of the cholinergic receptor protein of *Torpedo* electric tissue. Nature 229: 554–557.
28. Nevins, D. J. 1970. Relation of glycosidases to bean hypocotyl growth. Plant Physiol. 46: 458–462.
29. Newcomb, E. H. 1963. Cytoplasm-cell wall relationships. Annu. Rev. Plant Physiol. 14: 43–64.
30. Ridge, I. and D. J. Osborne. 1970. Hydroxyproline and peroxidases in cell walls of *Pisum sativum:* regulation by ethylene. J. Exp. Bot. 21: 843–856.
31. Riov, J. and M. J. Jaffe. 1973. A cholinesterase from bean roots and its inhibition by plant growth retardants. Experientia 29: 264–265.
32. Riov, J. and M. J. Jaffe. 1973. Cholinesterases from plant tissues. I. Purification and characterization of a cholinesterase from mung bean roots. Plant Physiol. 51: 520–528.