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THE DISTRIBUTION OF CHOLINESTERASES IN PLANT SPECIES*

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Abstract—The neostigmine-inhibited hydrolysis of acetylthiocholine has been found in extracts prepared from 23 species from five of the 24 families assayed: Characeae, Cruciferae, Graminae, Leguminosae, and Solanaceae but not present in 33 other species. In no case was activity found in all species tested from the five positive families. In extracts prepared from *Phaseolus aureus* Roxb. there was an agent which decolorizes the product formed in the colorimetric assay used for assaying cholinesterase. This decolorant is heat-inactivated and partially dialyzable.

INTRODUCTION

ACETYLCHOLINE[1,2] and a cholinesterase[3] have been identified in extracts prepared from tissues of *Phaseolus aureus*, the mung bean. In order to define further the role of these molecules in plants we have sought to localize cholinesterase activity in *P. aureus* and to determine its distribution in the plant kingdom. In *P. aureus*, cholinesterase activity has been identified in all of the organs examined;[4] and in the roots the enzyme is associated with the cell wall.[4] We now describe the results of a survey of a number of plant species which we have assayed for cholinesterase activity.

RESULTS

Survey of plants

Neostigmine-inhibited enzymatic hydrolysis of acetylthiocholine was found in 23 species from five families (Table 1): Characeae, Cruciferae, Graminae, Leguminosae and Solanaceae. Extracts prepared from other species of these families contained no detectable hydrolytic activity; and in *Zea mays* hydrolytic activity was identified in only some plant organs and appears to be absent from others.

Decolorizing activity

In assaying for cholinesterase activity, there are several reasons, besides its absence, that may account for a lack of demonstrable activity. One, is that the colored reaction product

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[1] JAFFE, M. J. (1970) *Plant Physiol* **46**, 768.

[2] JAFFE, M. J. (1972) in *Recent Advances in Phytochemistry* (RUNECKLES, V. C. and TSO, T. C., eds.), Vol. V, pp. 81–104, Academic Press, New York.

[3] RIOV, J. and JAFFE, M. J. (1973) *Plant Physiol.* **51**, 520.

[4] FLUCK, R. A. and JAFFE, M. J. (1974) *Plant Physiol.* **53**, 752.

TABLE 1. CHOLINESTERASE ACTIVITY OF EXTRACTS OF PLANTS

Plant	Plant part	Method of extraction	Activity
Characeae			
Nitella sp.	WP*	P†	−§
Aizoaceae			
Tetragonia expansa Murr.	L	P	−
Amaranthaceae			
Amaranthus retroflexus L.	WP	X,P	−
Betulaceae			
Alnus glutinosa (L.) Gaertn.	L	X	−
Caryophyllaceae			
Tunica saxifrage (L.) Scop.	L & S	X	−
Chenopodiaceae			
Salicornica virginica L.	S	X	−
Spinacia oleracea L.	L	P	−
Compositae			
Lactuca sativa L. cv. Grand Rapids	R	P	−
Tagetes patula L. cv. Early French Double	L	X	−
Crassulaceae			
Bryophyllum sp.	R	P	−
Crassula lycopodiodes Lam.	L	X	−
Cruciferae			
Brassica oleraceae L. var. *capitata* L.	L	X	−
Brassica hirta (L.) Moench cv. Tendergreen	R	P	−
Metthiola incana (L.) R. Br. cv. Trysomic Giant Imperial	L	X	−
Raphanus sativus L. cv. Red Prince	R	P	+
Cucurbitaceae			
Cucumis sativus L. cv. Marketmore	R	X,P	−
Euphorbiaceae			
Ricinus communis L. cv. Red Spire	R	P	−
Graminae			
Avena sativa L.	R, L	P	−
Hordeum volgare L. cv. Atlas 68	R, L	P	−
Zea mays L. Hulting X440	R	P	−
	L, R	X	−
	C‡, L‡	P	+
Haloragaceae			
Myriophyllum exalbescens Fern.	WP	X	−
Labiatae			
Coleus blumei Benth.	L	X	−
Leguminosae			
Albizia julibrissin (Willd.) Durazz	L, R	P	+
Cassia tora L.	R	P	+
	L	X	−
Cercis canadensis L.	F	X	−
Coronilla varia L.	R	P	+
Gleditsia triancanthos L.	L	X	+
Glycine max (L.) Merr. cv. Harosoy 63	R	P	+
Lathyrus latifolia L.	F	X	+
Lathyrus odoratus L.	R	P	+
Lens culinaris (L.) Medic.	R	P	+
Medicago sativa L. cv. Northwest	R	P	+
Mimosa pudica L.	R	P	+
Phaseolus vulgaris L. cv. Red Kidney	R	P	+
Pisum sativum L. cv. Alaska	R	X	+
P. sativum L. cv. Progress 9	R	X	+
Robinia pseudoacacia L.	L	X	−
Thermopsis carolininiana M. A. Curt	L	P	−

TABLE 1. *Continued*

Plant	Plant part	Method of extraction	Activity
Trifolium pratense L. cv. Kenland red	R	P	+
Trifolium repens L.	R	P	+
Trigonella Foenum-Graecum L.	R	P	+
Vicia faba L.	R	P	+
Vigna sinensis (L.) Savi	R	P	+
Lemnaceae			
Lemna perpusilla Torr. 6746 Landhok			
Liliaceae			
Allium cepa L. cv. Downing yellow globe	R	P	–
Palmae			
Cocos nucifera L.	E	X	–
Rosaceae			
Fragaria chiloensis Duchesne var. *ananassa* Bailey	L	X	–
Saxifragaceae			
Bergenia cordifolia (L.) Sternb.	L	X	–
Scrophulariaceae			
Antirrhinum majus L. cv. T scon	L	X	–
Solanaceae			
Capsicum frutescens L. var. *grossum* Bailey	L	X, P	–
Lycopersicon esculentum (L.) Mill. cv. Rutgers	R	X, P	+
Nicotiana alata (Moore and Otto) Link and Otto var. *grandiflora* Comes	L	X	–
Petunia hybrida Vilm.	L	X	–
Solanum melongena L. cv. Black Beauty	L, R	X	+
S. tuberosum L.	L	X	+
Umbelliferae			
Daucus carota L. var. *sativa* DC	L	X	–
Urticaceae			
Laportea canadensis Gaudich.	L	X	–

* E = endosperm; F = flowers; L = leaves; R = roots; S = stems; WP = whole plant.
† X = extracted according to experimental; P = particulate fraction prepared according to experimental.
‡ Dark-grown plants.
§ See Note added in proof.

2-nitro-5-thiobenzoate, which is formed during the assay for activity might be decolorized by a component present in the extract. Such a decolorizing component has now been found in *Phaseolus aureus*. The addition of an aliquot of root extract from *P. aureus* to a solution containing 2-nitro-5-thiobenzoate results in a rapid bleaching of the solution (Fig. 1): within 5 min the A_{412} of the solution decreased to 25% of the original value. The decolorant activity was destroyed by heat and decreased following dialysis. Pre-incubation of the extract with 50 μM neostigmine bromide for 30 min at 37° did not affect the rate or extent of bleaching.

DISCUSSION

The hydrolysis of choline esters by extracts of various plants has been described in other reports.[5–7] In many of these studies, however, no effective controls were used to identify or characterize the enzyme which caused the hydrolysis. The criterion that has often been

[5] JANSEN, E. F., NUTTING, M. D. F. and BALLS, A. K. (1948) *J. Biol. Chem.* **175**, 975.
[6] MOUNTER, L. A. and MOUNTER, M. E. (1962) *Biochem. J.* **85**, 576.
[7] SCHWARZ, H. M., BIEDERON, S. I., VON HOLDT, M. M. and REHM, S. (1964) *Phytochemistry* **3**, 189.

used for demonstrating cholinesterase activity in plant extracts[5-9] is its inhibition by physostigmine, a potent inhibitor of animal cholinesterases.[10,11] This is probably an unfortunate choice because physostigmine is a relatively poor inhibitor of mung bean cholinesterase. In some cases[5,6] the choline esters were probably hydrolyzed by general esterases. However, the characteristics of the hydrolysis of acetylcholine by these enzyme preparations differ sharply from those observed with cholinesterase from *P. aureus* with respect to such properties as optimal substrate concentration and K_m. The cholinesterase from *P. aureus* displays substrate inhibition with 100% inhibition at substrate concentrations

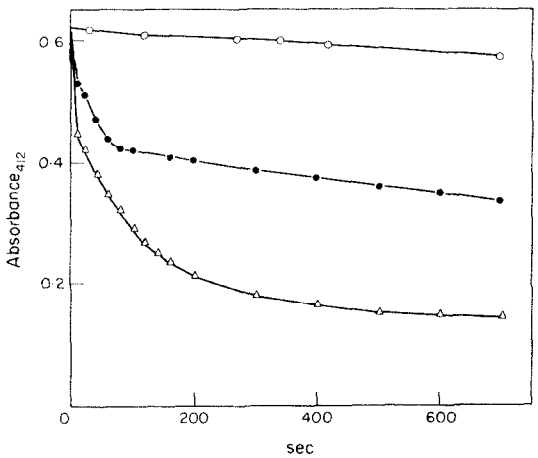

FIG. 1. DECOLORANT FROM ROOTS OF *Phaseolus aureus*.
The root extract and the solution containing 2-nitro, 5-thiobenzoate were prepared as described in the experimental section. The decrease in A_{412} which follows the addition of root extract (△—△) is completed in less than 10 min and is prevented by first heating the root extract (○—○). Both the rate and the extent of bleaching are decreased following dialysis (●—●), but there is no further loss of activity if the extract is further dialyzed. Root extract, △—△; root extract heated in boiling water bath for 30 min, ○—○; root extract dialyzed 1× or 2× against 400 vol. of 0·01 M K phosphate, pH 7·0, ●—●; H_2O only, +.

greater than 10 mM.[3] However, a commercial preparation of wheat germ esterase was reported to hydrolyze acetylcholine at a substrate concentration of 1 M.[6] Furthermore, the K_m of acetylcholine for the cholinesterase from *P. aureus* and for citrus acetyl esterase are 72 μM[3] and 1·6 M[5], respectively.

In this work we have used as criteria for cholinesterases their inhibition by neostigmine, their localization in the cell wall, and a special extraction procedure, all of which were found to characterize the enzyme from *P. aureus*.[3] The hydrolysis of acetylthiocholine, which is inhibited by 25 μM neostigmine bromide, has been found in 23 species in five families. The enzyme in *Raphanus sativus* may be related to the sinapine esterase extracted

[8] FOTTRELL, P. F. (1968) *Phytochemistry* **7**, 23.
[9] VAN DER JOOSTE, J. and MORELAND, D. E. (1963) *Phytochemistry* **2**, 263.
[10] AUGUSTINSSON, K. (1960) in *The Enzymes* (BOYER, P. D., LARDY, H. and MYRBACK, K., eds.), Vol. IV, pp. 521–540, Academic Press, New York.
[11] RICHTER, D. and CROFT, P. G. (1942) *Biochem. J.* **36**, 746.

from *Brassica hirta*, which has been shown to hydrolyze acetylcholine.[12] The presence of cholinesterase activity in *Nitella flexilis* has been reported[13] but our findings are equivocal (Table 1). It is interesting to note that in the stinging nettle, whose toxic principle is a mixture of acetylcholine and histamine[14] and which contains an enzyme for synthesizing acetylcholine,[15] no demonstrable cholinesterase activity has been found. Nor is any demonstrable cholinesterase activity present in the closely related nettle, *Laportea canadensis* (Table 1).

The absence of cholinesterase activity from many plant preparations does not necessarily imply that the enzyme is not present in the intact plant. For example, the enzyme activity could be lost during the preparation of the extracts either by irreversible inhibition by compounds in the tissue[16–18] or by extraction or fractionation procedures that are ideal for *P. aureus* but not for other species. However, it should be noted that we have identified cholinesterase activity in the leaves of *Solanum tuberosum* which have been shown to contain an inhibitor of cholinesterase activity.[19,20] Another possible explanation for the apparent absence of cholinesterase activity from some plant extracts is that the enzyme may be present in amounts below the level of detection of the assay. In general, plants containing 100-fold less cholinesterase activity (on a fresh weight basis) than *P. aureus* would have enzyme levels not detectable by our method. The presence of decolorant activity in a tissue extract could also minimize the apparent amount of cholinesterase activity present. In addition to the decolorizing agent(s) reported here from beans, a decolorant of 2-nitro-5-thiobenzoate is also present in sea urchin extracts;[21] and although it is also destroyed by heating, it is completely dialyzable.

When we began this survey of plant species, we had hoped that the distribution of cholinesterase in the plant kingdom might provide an insight into the role of acetylcholine in plant metabolism. However, the presence of the activity in a few widely dispersed plant groups and its apparent absence from other members of these same groups make a functional interpretation on this basis impossible at present. We have tentatively identified the hydrolytic activity in *Zea mays*, *Nitella* sp., and the solanaceous plants as cholinesterases. However, the properties of these enzymes will have to be investigated in more detail before we can conclusively identify them.

EXPERIMENTAL

Plants. Lemna perpusilla Torr. 6746 Landhok and *Hordeum vulgare* L. cv. Atlas 68 were provided by Dr. Jerry McClure, Miami University, Oxford, Ohio; seeds of *Amaranthus retroflexus* L. were a gift from Dr. R. E. Holm, Diamond Shamrock Corporation, Painesville, Ohio; *Zea mays* L. Hulting X440 was provided by C. E. Fluck, Hulting Hybrid Corn Co., Geneseo, Illinois, Nettle (*Laportea canadensis* Gaudich.) leaves, redbud (*Cercis canadensis* L.) flowers, evalasting sweet pea (Lathyrus latifolia L.) flowers, and *Myriophyllum exalbescens* L. were collected locally. *Nitella* sp. was purchased from Carolina Biological Supply Co., Burlington, North Carolina. Seeds of other plants were purchased from commercial sources. The seeds were either sown in vermiculite and placed

[12] TZAGALOFF, A. (1963) *Plant Physiol.* **38,** 207.
[13] DETTBARN, W. D. (1962) *Nature* **194,** 1175.
[14] EMMELIN, N. and FELDBERG, W. (1947) *J. Physiol.* **106,** 440.
[15] BARLOW, R. B. and DIXON, R. O. D. (1973) *Biochem. J.* **132,** 15.
[16] ANDERSON, J. W. (1968) *Phytochemistry* **7,** 1973.
[17] HAISSIG, B. E. and SCHIPPER, A. L., JR. (1972) *Analyt. Biochem.* **48,** 129.
[18] ORGELL, W. H., VAIDYA, K. A. and DAHM, P. A. (1958) *Science* **128,** 1136.
[19] HARRIS, H. and WHITTAKER, M. (1962) *Ann. Hum. Genet., Lond.* **26,** 73.
[20] STREET, H. E., KENYON, A. E. and WATSON, G. M. (1946) *Ann. Appl. Biol.* **33,** 1.
[21] WOLFSON, N. (1972) *J. Embryol. Exp. Morphol.* **28,** 511.

in growth chambers (16 hr photoperiod, 24d) or sown in soil and placed in the greenhouse. *Lemna perpusilla* was grown in axenic culture in Hutner's medium. The buffer used was 10 mM K phosphate, pH 7·0, unless otherwise specified.

Preparation of extracts. The extraction employed was that described for extracting cholinesterase from *P. aureus*[3,4] and was performed at 4°. Plant parts were homogenized in 2 vol (v/w) of buffer in a Virtis homogenizer, and the homogenate was stirred for 30 min and filtered through two layers of a 54 μm mesh nylon net. The residue was resuspended in 2 vol. of 4% $(NH_4)_2SO_4$ in buffer, and dispersed in a Virtis homogenizer. The homogenate was stirred for 60 min, filtered through nylon net, and centrifuged for 15 min at 20000 *g*. Solid $(NH_4)_2SO_4$ was added to the supernatant up to 80% saturation and the precipitate collected by centrifugation and dissolved in buffer. This solution was dialyzed against buffer overnight and the dialysate was centrifuged to clarify the extract.

To obtain a particulate fraction, plant tissues were homogenized in buffer with a Virtis homogenizer and the homogenate centrifuged at 27000 *g* for 10 min. The supernatant fluid was discarded and the pellet was resuspended in buffer and recentrifuged. The supernatant fluid was again discarded and the pellet was resuspended in buffer and used.

Cholinesterase assays. The assay for cholinesterase was a modification[3,4] of the colorimetric assay described by Ellman *et al.*[22] Control assays contained 25 μm neostigmine bromide.

Preparation and assay of decolorant. Roots from *P. aureus* were homogenized in buffer and the homogenate was centrifuged for 2 hr at 27000 *g*. The pellet was discarded and the supernatant fluid was filtered through 2 Whatman GF/B glass fiber filters. The filtrate was centrifuged for 2 hr at 27000 *g* and the pellet discarded. One aliquot of the final supernatant fluid was dialyzed against 400 vol. of buffer; and another aliquot was heated at 100° for 30 min. Both the dialysate and the heated aliquot were centrifuged before assaying. The colored solution was prepared by adding 0·75 ml of 12·5 mM acetylthiocholine chloride and 0·75 ml of 2·6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to 20 ml of 0·25 M K phosphate, pH 8·0, and incubating the mixture at 37°. The base-catalyzed hydrolysis of acetylthiocholine results in the release of acetate and thiocholine, and the reaction of thiocholine with DTNB leads to the formation of 2-nitro, 5-thiobenzoate, a yellow anion with an absorption maximum at 412 nm. To 2·5 ml of the colored solution ($A_{412} \cong 1·2$) in a cuvette was added 2·5 ml of the tissue extract and the decrease in A_{412} was recorded.

* *Note added in proof.* The initial reaction was +ve but after the submission of this manuscript, we purchased another batch of *Nitella* sp. from Carolina Biological Supply Co. in order to characterize the cholinesterase-like activity in this organism. However, the activity in this second batch was very low compared to that of the first batch, and the small amount of activity which was present was similar in certain respects to cholinesterases from animal tissues. Therefore, it is possible that the cholinesterase activity in our preparations of *Nitella* is a result of contamination by animals - either eggs, zygotes, small larvae, or small adult invertebrates.

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[22] Ellman, G. L., Courtney, K. D., Andres, V., Jr. and Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88.