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A STUDY OF SOME PLANT ESTERASES

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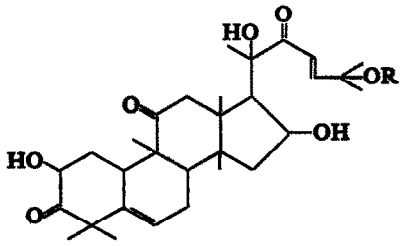
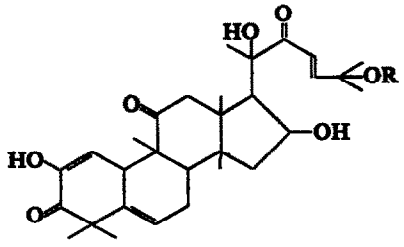
Abstract—High carboxylic ester hydrolase (esterase) activity towards indophenyl acetate was found in green beans, cabbage, potato tuber, citrus albedo and flavedo, and fruits of many cucurbits. Only potato and cucurbits hydrolysed esters of cucurbitacins, bitter principles occurring in the Cucurbitaceae, and cholinesterase activity was found only in citrus and in one variety of cucurbit. Starch gel electrophoresis showed that all extracts contained a multiplicity of esterases active against α-naphthyl acetate which differed in different species, in different strains of the same species and even in different parts of the same plant. Several cucurbitacin esterases were also detected in potato and cucurbit extracts, but citrus contained only one cholinesterase.

INTRODUCTION

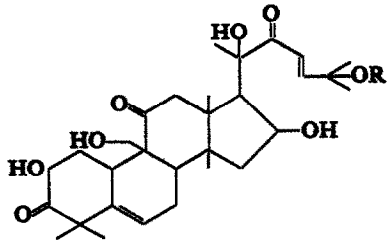
REHM, Enslin, Meeuse and Wessels[1] isolated a series of bitter principles which they called cucurbitacins, from members of the Cucurbitaceae. They found evidence for the existence of two primary cucurbitacins, E and B (Fig. 1), and postulated that others are formed from them during growth of the plant. This view was substantiated by the discovery that fruit juices from many cucurbits are capable of enzymic transformation of one bitter principle to



Cucurbitacin B: R = OAc
Cucurbitacin D: R = H



Cucurbitacin E: R = OAc
Cucurbitacin I: R = H



Cucurbitacin A: R = OAc
Cucurbitacin N: R = H

FIG. 1. STRUCTURE OF SOME CUCURBITACINS.

[1] S. REHM, P. R. ENSLIN, A. D. J. MEEUSE and J. H. WESSELS, *J. Sci. Food Agric.* **8**, 679 (1957).

another. One of these enzymes which converts cucurbitacin B to D, E to I, and A to N appeared to be a carboxylic ester hydrolase (esterase).

While animal esterases have been studied extensively, the esterases of higher plants have received scant attention. Most work has been done on citrus fruits which contain an acetylesterase capable of hydrolysing a wide range of esters.[2,3] Acetylesterase activity was also found in wheat germ[4,5] and in the tomato and lucerne.[6] Substrate specificity tests on the last two and on citrus, however, indicated that the activity was not due to a single enzyme.[6] Recently starch gel electrophoresis has been employed to study the esterases of maize kernels,[7,8] wheat seeds and cucumber and soybean seedlings,[8] all of which showed more than one such enzyme.

The primary purpose of the present study was to investigate the esterases occurring in plants belonging to the Cucurbitaceae. At the same time a study of esterases in other plant families, particularly from the point of view of their ability to hydrolyse cucurbitacin acetates, was undertaken.

RESULTS

A preliminary survey of the esterase activity of a number of plants belonging to the Cucurbitaceae and other families was made, using indophenyl acetate as substrate. This compound was selected because aromatic esters have been reported to be attacked by a wide range of esterases,[9] and also because they lend themselves to the use of rapid and sensitive colorimetric methods of enzyme assay. Of the esters frequently used, *o*-nitrophenyl-acetate[4] and 2-azobenzene-1-naphthyl acetate[10] were found to be unsuitable because of their very low solubility in water. Organic solvents could not be used in the present study because the cucurbit esterases were partially inhibited by small amounts (2%) of ethanol or acetone. Indophenyl acetate,[11] on the other hand, was sufficiently soluble in 0·5% ethanol to give a satisfactory assay method.

The esterase activities of a number of cucurbits are shown in Table 1. The fruits of all species examined showed some activity, the values ranging from 27 units/ml of juice (see Experimental) in a variety of *Cucurbita pepo* to 3180 units/ml in *Luffa acutangula* var. *amara*. The activity of *Cucumis myriocarpus* showed a marked seasonal variation; it was highest in November when the fruits were green and decreased to almost zero in January when they were fully ripe. In *Cucurbita maxima cv.* Green Hubbard, on the other hand, no relation was found between activity and maturity of fruit from the same plant. The range in activity of 119–845 units/ml found in commercial samples of this variety examined over a period of one year also showed no seasonal trend and was possibly due to genetic differences in the plants. In five cases the leaves and stems were also examined for esterase activity which was generally present at a lower level than in the fruit. An exception was *Ecballium elaterium* where the highest activity occurred in the leaves. The stems were always the least active.

[2] A. SOSA and C. SANNIE, *Bull. Soc. Chim. biol. Paris* **26**, 457 (1944).
[3] E.F. JANSEN, R. JANG and L. R. MACDONNELL, *Arch. Biochem.* **15**, 415 (1947).
[4] E. F. JANSEN, M. D. F. NUTTING and A. K. BALLS, *J. biol. Chem.* **175**, 975 (1948).
[5] L. A. MOUNTER and M. E. MOUNTER, *Biochem. J.* **85**, 576 (1962).
[6] L. R. MACDONNELL, R. JANG, E. F. JANSEN and H. LINEWEAVER, *Arch. Biochem.* **28**, 260 (1960).
[7] D. SCHWARTZ, *Proc. Natl. Acad. Sci.* **46**, 1210 (1960).
[8] J. VAN DER W. JOOSTE and D. E. MORELAND, *Phytochemistry* **2**, 263 (1963).
[9] D. K. MYERS, *The Enzymes*, Ed. P. D. BOYER, H. LARDY and R. MYRBÄCK, Vol. 4, p. 475, Academic Press, New York (1960).
[10] J. EPSTEIN, M. DEMEK and V. C. WOLFF, *Analyt. Chem.* **29**, 1050 (1957).
[11] D. N. KRAMER and R. M. GAMSON, *Analyt. Chem.* **30**, 251 (1958).

The highest esterase activity in the non-cucurbits examined was found in the cabbage (head), green bean (fruit), carrot (root), potato (tuber) and citrus (albedo and flavedo) (Table 2). In agreement with Jansen *et al.*[3] we found very little activity in the juice of citrus fruits.

TABLE 1. DISTRIBUTION OF ESTERASES IN CUCURBITACEAE

(Extracts prepared as described in Experimental; enzyme assayed by indophenyl acetate method.)

Species*	Portion of plant	Enzyme activity† (units/ml)
Cucurbita pepo L.		
cv. "Little Gem"	Fruit	272
cv. "Long White Bush"	Fruit	119
cv. "Long Green Bush"	Fruit	27
cv. "White Custard"	Fruit	34
cv. "Golden Custard"	Fruit	247
cv. "Table Queen"	Fruit	43
cv. "Grahamstown Marrow"	Fruit	71
Cucurbita sororia Bail.‡	Fruit	105
Cucurbita mixta Pang.		
cv. "Japanese Pie"	Fruit	118
cv. "Tennessee Sweet Potato"	Fruit	144
Cucurbita maxima Duch.		
cv. "Green Hubbard"	Fruit	119–845 (51)
Cucurbita andreana Naud.		
ex Whitaker‡	Fruit	669
	Leaves	92
	Stem	10
ex Parodi‡	Fruit	198
ex Parodi	Fruit	218
Cucurbita moschata Duch.		
cv. "Butternut"	Fruit	357
Luffa acutangula (L.) Roxb.	Fruit	533
	Leaves	92
	Stem	49
var. *amara*‡	Fruit	3180
	Leaves	133
	Stem	125
Luffa operculata Cogn.	Fruit	59
Ecballium elaterium (L.) A. Rich.‡	Fruit	144
	Leaves	158
	Stem	76
Cucumis heptadactylus Naud.‡	Fruit	620
	Leaves	187
	Stem	82
Cucumis angolensis Hook. f. ex Cogn.‡	Fruit	61
Cucumis dinteri Cogn.‡	Fruit	771
Cucumis africanus L. f.	Fruit	430
Cucumis anguria L.	Fruit	57
Cucumis myriocarpus Naud.‡	Fruit	28–2675 (5)
Cucumis melo L.		
Breeding line HB 45/3	Fruit	33–297 (28)
Momordica foetida Schum. & Thonn.	Fruit	570
Trichosanthes anguina L.	Fruit	280

* Names of *Cucumis* species as interpreted by Meeuse.[12]

† Figures in brackets refer to numbers of samples examined.

‡ Bitter variety.

[12] A. D. J. MEEUSE, *Bothalia* **8**, 1 (1962).

For the detection of cucurbitacin esterase activity, the plant extracts were incubated with elaterinide (cucurbitacin E glycoside) and the products of the reaction were further hydrolysed with a β-glucosidase and the resulting aglycones identified by chromatography. The presence of cucurbitacin I or D (dihydro-cucurbitacin I) was taken as evidence of deacetylation of the substrate. All the cucurbits which showed activity towards indophenyl acetate deacetylated elaterinide. Potato juice converted cucurbitacin E quantitatively to I. In contrast, no traces of deacetylated products were detected after treatment of elaterinide with bean or cabbage extract, but extensive destruction of the cucurbitacin molecule occurred as judged by the decrease in intensity of the cucurbitacin E spot after 2 hr incubation; a similar breakdown of

TABLE 2. DISTRIBUTION OF ESTERASES IN FAMILIES OTHER THAN CUCURBITACEAE
(Extracts prepared as described in Experimental; enzymes assayed by indophenyl acetate method.)

Family and species	Portion of plant	Enzyme activity units/ml
Cruciferae		
Cabbage, *Brassica oleracea* var. *capitata*	Head	368–646 (6)
Rosaceae		
Apple, *Malus domestica*	Fruit	10
Peach, *Prunus persica*	Fruit	19
Leguminosae		
Green bean, *Phaseolus vulgaris*	Fruit	409–1079 (5)
Anacardiaceae		
Mango, *Mangifera indica*	Fruit	35
Umbelliferae		
Carrot, *Daucus carota*	Root	254
Solanaceae		
Tomato, *Lycopersicum esculentum*	Fruit	5
Egg-fruit, *Solanum melongena*	Fruit	63
Potato, *Solanum tuberosum*	Tuber	204–1157 (6)
Liliaceae		
Onion, *Allium cepa*	Bulb	46
Bromeliaceae		
Pineapple, *Ananas sativus*	Fruit	0
Rutaceae		
Orange, *Citrus sinensis*	Juice	8
	Albedo	84–402 (2)
	Flavedo	276–552 (2)

elaterinide has been observed after prolonged incubation with some cucurbit juices. Orange albedo and flavedo extracts caused neither deacetylation nor destruction of cucurbitacin E, even after prolonged incubation.

The activities of the crude extracts towards a number of other esters were studied by measuring manometrically the CO_2 released from bicarbonate by the acid produced. Because of low activity of the extracts, the volumes of CO_2 evolved in many cases were too small to permit accurate measurements of the relative activities towards different substrates. The results (Table 3) are therefore considered as qualitative only. Nevertheless they show that plants vary considerably in their ability to hydrolyse different esters. Two points are of particular interest. The results with cucurbitacin A and elaterinide confirmed that activity towards this class of ester is found only in cucurbits and potato. The lack of acid production

TABLE 3. HYDROLYSIS OF VARIOUS ESTERS BY PLANT EXTRACTS

(Enzyme activity determined manometrically as described in Experimental.)

Source of extract	*o*-Nitro phenyl acetate	acetyl salicylic acid	Tri-acetin	Tri-propionin	Tri-butyrin	Ethyl acetate	Tween 20	Cucurbi tacin A	Elateri nide	Acetyl choline bromide
Cucurbita maxima										
cv. Green Hubbard, fruit	+	+	+	+	+	0	+	+	±	+
Cucumis myriocarpus, fruit	+	±	+	+	+	0	0	+	*	0
Cucumis melo, fruit	+	+	+	+	+	+	±	+	+	0
Potato	+	±	+	+	+	0	0	±	+	0
Bean	+	+	+	+	+	0	0	0	0	0
Cabbage	+	+	+	+	+	0	0	0	0	0
Lemon flavedo	+	+	+	±	0	+	+	0	0	±
albedo	+	0	+	+	±	0	±	0	0	+
Grapefruit flavedo	+	+	+	+	+	0	0	0	0	±
albedo	+	±	+	+	±	0	0	0	0	0
Orange flavedo	+	+	+	+	+	±	+	0	0	±
albedo	+	±	+	+	±	0	±	0	0	±

0 = Less than 5 μl CO_2 produced. ± = 5–10 μl CO_2. + = More than 10 μl CO_2.

* Could not be tested because of presence of elaterase.

by cabbage and bean extracts showed that the destruction of the cucurbitacin molecule which was detected chromatographically was not preceded by deacetylation. Acetylcholinesterase activity was also of limited distribution, being found only in the fruit of the Green Hubbard, the flavedo of the three citrus fruits and orange and lemon albedo.

The number and types of esterases present in the plants were studied further by starch gel electrophoresis. Figures 2–4 show some typical zymograms. Most extracts contained several enzymes capable of splitting α-naphthyl acetate. The enzymes were different in different

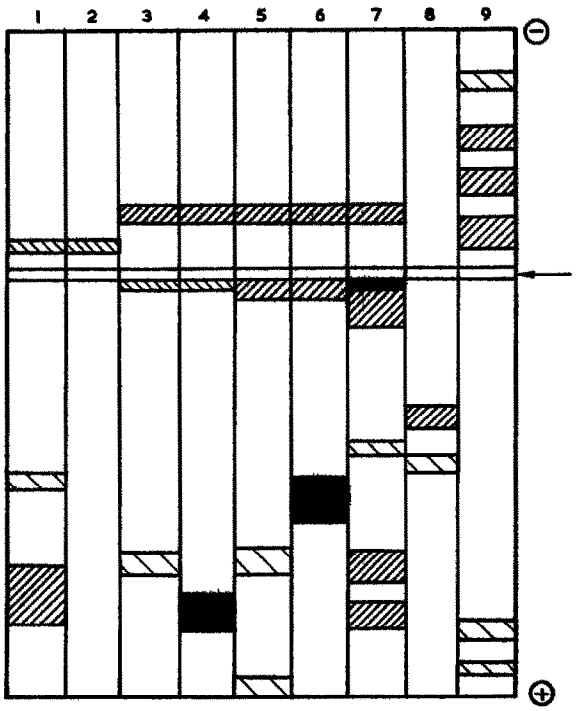


FIG. 2. ESTERASE ZYMOGRAMS OF SOME PLANT EXTRACTS.

Stained with α-naphthyl acetate plus tetrazotized *o*-dianisidine. Intensity of bands indicated by shading. Arrow shows point of application of sample.

1. Fruit of *Cucurbita andreana* (strain from Whitaker). 2. Fruit of *Cucurbita andreana* (strain from Parodi). 3. *Cucumis melo* fruit. 4. *Cucumis myriocarpus* fruit. 5. *Cucurbita maxima*, cv. Green Hubbard fruit. 6. Potato tuber. 7. Green bean. 8. Cabbage. 9. Maize kernel.

species and even in different parts of the same plant. Different batches of material from commercial sources also showed differences in esterase pattern. For example, some potatoes lacked the esterase which moved towards the cathode, shown in Fig. 2, while extracts of orange, lemon and grapefruit albedo showed from one to four acid esterase bands, the intensity of which, relative to that of the most slowly moving, varied from sample to sample. These variations are possibly related to differences in the genetic constitution of the plants, since the two strains of *Cucurbita andreana* which were examined showed markedly different esterase patterns (Fig. 2). Genetic control of the nature of the esterases present in maize kernel has also been reported.[7]

Activity towards 6-bromo-2-naphthylcarbonaphthoxy choline iodide was detected only in the zymograms of the albedos and flavedos of the three citrus species. Even here, not all

batches of material were equally active, since several samples of orange and lemon flavedo were encountered which failed to show any cholinesterase band. No cholinesterase activity was detected in the zymograms of extracts of Green Hubbard fruits. This is in contrast to the results of the manometric test carried out on different samples of fruit which showed slight activity towards acetylcholine (Table 3), and may indicate that the enzyme is much more active towards acetylcholine than towards other choline esters. Alternatively, the activity may vary, as in citrus, depending on the breeding line or maturity of the fruit.

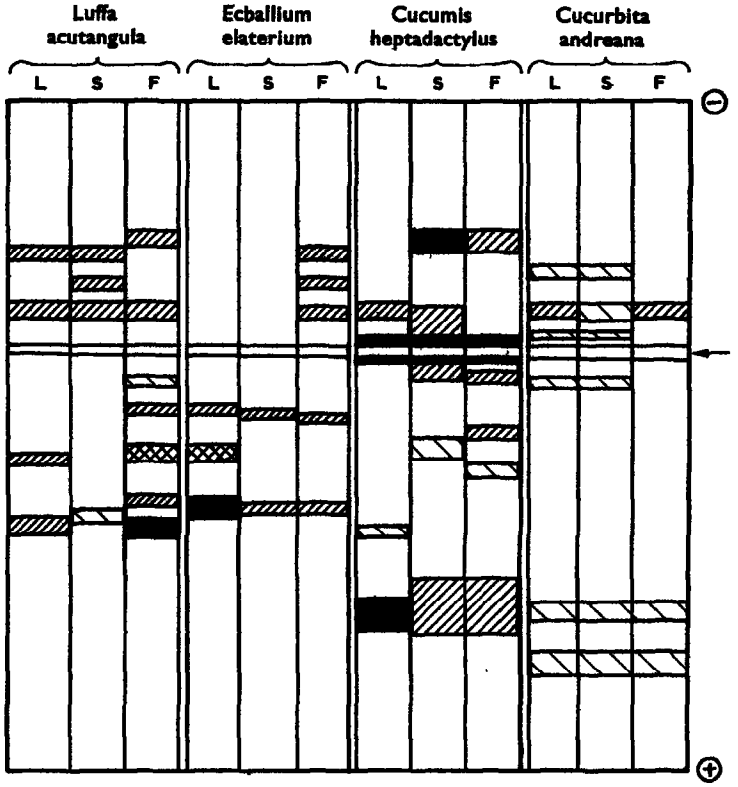


FIG. 3. ZYMOGRAMS OF EXTRACTS OF LEAF (L), STEM (S) AND FRUIT (F) OF SOME CUCURBITS. Stained with α-naphthyl acetate plus tetrazotized *o*-dianisidine.

Each of the citrus extracts showed only one cholinesterase band which appeared from its position on the zymogram to be the same in the albedos and flavedos of all three species (Fig. 4). Further evidence of the identity of the cholinesterases of the different citrus tissues was obtained from a study of the effect of eserine and DFP on their activity. All were unaffected by 10^{-5} M di-isopropylfluorophosphate (DFP) and slightly stimulated by 10^{-5} M eserine.

In the zymograms of the flavedo extracts, the position of the cholinesterase band did not coincide with any of the bands revealed with α-naphthyl acetate (Fig. 4). The cholinesterase is therefore unreactive towards this substrate. The cholinesterase of the albedo extracts, on the other hand, occupied the same position as one of the α-naphthylacetylesterases, but,

whereas hydrolysis of the choline ester was unaffected by 10^{-5} M DFP, that of α-naphthyl acetate was partially inhibited, suggesting that different enzymes are involved.

Figure 5 shows the distribution of cucurbitacin esterases on starch gel electropherograms of extracts of potato, Green Hubbard and *Cucumis melo* fruit. The bands are somewhat diffuse, particularly in the potato. It is not possible to state with certainty how many enzymes hydrolysing this class of compound are present, but it is evident that there are several in each

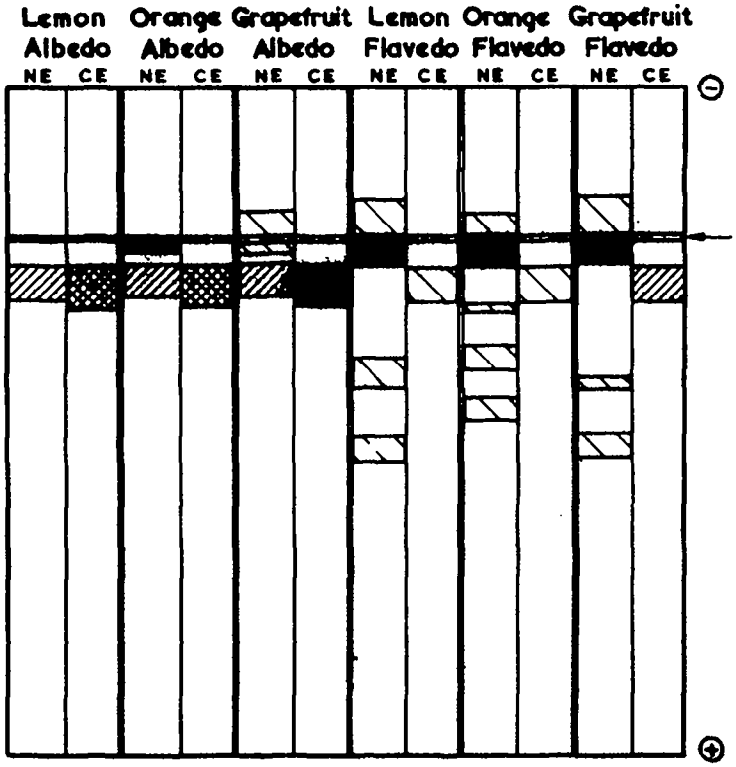


FIG. 4. ZYMOGRAMS OF EXTRACTS OF CITRUS ALBEDO AND FLAVEDO STAINED FOR α-NAPHTHYLACETYLESTERASE (NE) AND CHOLINESTERASE (CE).

plant. The positions of many of the bands do not correspond with those revealed with α-naphthyl acetate suggesting that the cucurbitacin esterases have little or no activity towards aromatic esters.

DISCUSSION

The present work shows that plants contain a multiplicity of esterases which differ in different species, in different strains of the same species, and even in different parts of the same plant. This is similar to the state of affairs which exists in animal tissues.[13,14]

It is not possible to decide how many of the plant esterases distinguishable by starch gel electrophoresis are isozymes and how many differ in substrate specificity and other biochemical characteristics. The cholinesterase of the citrus flavedos clearly differs in substrate specifi-

[13] J. PAUL and P. FOTHERILL, *Biochem. J.* 78, 418 (1961).
[14] J. M. ALLEN and R. L. HUNTER, *J. Histochem. Cytochem.* 8, 50 (1960).

city and sensitivity to inhibitors from the enzymes responsible for the hydrolysis of α-naphtyl acetate in these tissues. Similarly, some if not all of the cucurbitacin esterases in cucurbit fruits and potato are distinct from the α-naphthylacetylesterases. Even amongst the latter some differentiation may be made on the basis of sensitivity towards inhibition by DFP and *p*-choloromercuribenzoic acid (PCMB). It is thus clear that attempts to characterize a plant enzyme as an esterase or a lipase as done recently by Mounter and Mounter[5] or to classify it as an A, B or C esterase according to Augustinsson[15] are only justified when the enzyme has been isolated in a pure state.

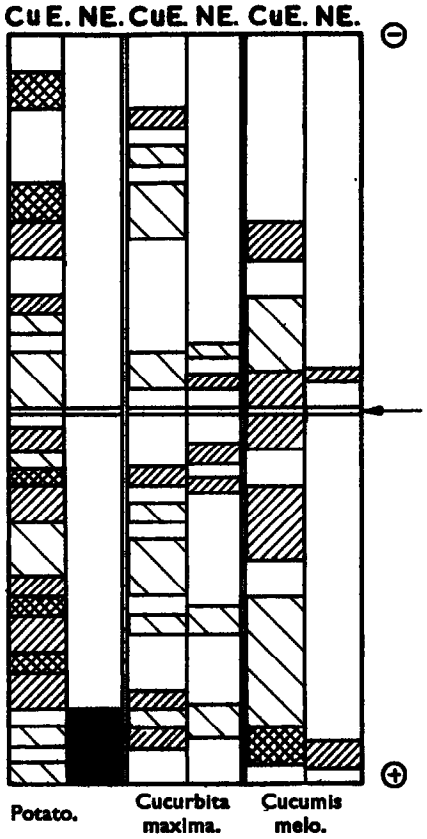


FIG. 5. DISTRIBUTION OF CUCURBITACIN ESTERASE (CuE) AND α-NAPHTHYLACETYLESTERASE (NE) IN ZYMOGRAMS OF POTATO TUBER, *Cucurbita maxima*, CV. GREEN HUBBARD AND *Cucumis melo* FRUIT.

Nothing is known of the function of any of these esterases in the plant. Although cucurbitacin esterases are found most frequently in members of the Cucurbitaceae, they do not appear to be associated with the presence of cucurbitacins, since they are found in both bitter and non-bitter varieties and they are also produced in the potato in which these compounds are not known to occur. In this respect the esterases differ from the β-glycosidase elaterase, which is unique in its high activity towards cucurbitacin glycosides and has been found only in bitter cucurbits.[16]

[15] K. AUGUSTINSSON, *Nature, Lond.* **181**, 1786 (1958).
[16] P. R. ENSLIN, F. J. JOUBERT and S. REHM, *J. Sci. Food Agric.* **7**, 646 (1956).

The function of cholinesterase in plants is also obscure. We have found cholinesterase activity only in citrus albedo and flavedo and in the fruit of *Cucurbita maxima*, cv. Green Hubbard. Other workers have reported it also in wheat germ[4,5] and lucerne.[6] Our experience has shown that even in the species where it is found, it is not invariably present. Acetylcholine however occurs in many plants, including the potato,[17] in which we have failed to detect the hydrolase. Phosphocholine and glycerophosphocholine, products of the enzymic hydrolysis of lecithin, have also been found in many plant tissues.[17] If the function of cholinesterases in plants is related to the hydrolysis of these esters, it would be expected that their presence would be more universal than indicated by the present survey.

Some of the carboxylic ester hydrolase activity of plant tissues may be due to proteinases or peptidases which can hydrolyse a number of esters in addition to peptides. An understanding of the function of the different esterases *in vivo* must thus wait on fuller knowledge of the substrate specificity of the purified enzymes.

EXPERIMENTAL

Substrates

Triacetin, tripropionin, tributyrin, *o*-nitrophenyl acetate, α-naphthyl acetate, acetylsalicylic acid and ethyl acetate were all of analytical purity. Acetylcholine bromide was obtained from L. Light and Co. Ltd, 6-bromo-2-naphthylcarbonaphthoxy choline chloride from Dajac Laboratories, and Tween 20 (polyoxyethylene lauryl alcohol) from Rohm and Haas Co. Cucurbitacin A and elaterinide, which were chromatographically pure, were supplied by Dr. P. R. Enslin of this laboratory. Indophenyl acetate was prepared and purified according to Kramer and Gamson.[11]

Inhibitors

Eserine salicylate was obtained from E. Merck, DFP from L. Light and Co. Ltd, and PCMB from Hopkin and Williams Ltd.

Enzyme Extracts

Citrus albedo and flavedo extracts were prepared by the method of Jansen *et al.*[3] Other fruits and vegetables were peeled where necessary and the flesh was minced and squeezed through cheese-cloth. The juice was centrifuged for 30 min at 2500 *g* at 5° and the supernatant used as a source of crude enzyme. For electrophoresis on starch gel the extract was usually concentrated fivefold by treatment with Sephadex G–25 (AB Pharmacia, Uppsala, Sweden).

Measurement of Enzyme Activity

(*a*) *Indophenyl acetate method.* A modification of the procedure of Kramer and Gamson[11] was used. Indophenyl acetate (12·1 mg) in 1·5 ml ethanol was diluted immediately before use to 100 ml with 0·067 M phosphate buffer, pH 6·5. This solution (5 ml) was added to the enzyme extract (5 ml) giving a substrate concentration in the final reaction mixture of $1{\cdot}67 \times 10^{-4}$ M. After incubating the mixture at 30° for 15 min, 0·05 M Na_2CO_3 and water (5 ml) were rapidly added to bring the pH to 8·0 and the colour produced was immediately measured in an Evelyn colorimeter at 620 mμ. A blank, containing water instead of enzyme, was run simultaneously to correct for non-enzymic hydrolysis of the substrate.

[17] M. GUGGENHEIM, *Die Biogenen Amine*, p. 111, S. Karger, Basel (1951).

Kramer and Gamson[11] carried out their reaction at pH 8·0 and 20°. Owing to the high ambient temperature we were compelled to work at 30°, at which temperature considerable non-enzymic hydrolysis of the substrate occurred at pH 8·0 but not at pH 6·5. The incubation was therefore carried out at pH 6·5 but the mixture was adjusted to pH 8·0 before taking colorimetric readings in order to measure the absorption of the indophenylate ion.

Under the conditions described, a zero-order reaction was obtained over a range of optical density readings of 0·05–0·45. The unit of enzyme activity was defined as the amount of enzyme which, under the conditions of the test, gave an optical density of 0·01.

(*b*) *Enzymes hydrolysing acetylated cucurbitacins.* These were detected by two methods:

(i) In crude plant extracts. Enzyme extract (25 ml) was added to a 3% solution of elaterinide in ethanol (1 ml) and the mixture was incubated at 30° for 2 hr. Proteins and gums were precipitated by the addition of ethanol (25 ml) and saturated basic lead acetate (25 ml). Excess of lead acetate was removed from the supernatant with saturated KH_2PO_4 (20 ml). Cucurbitacins were extracted three times with $CHCl_3$ (8 ml), which was removed by distillation. The residue was dissolved in ethanol (1 ml), water (25 ml) was added and the mixture was incubated at 30° for 2 hr with elaterase[18] (2–10 mg crude enzyme in 5 ml water) to hydrolyse the cucurbitacin glycosides. The aglycones were extracted with $CHCl_3$ and identified by paper chromatography.[1]

(ii) In extracts prepared from starch gel electropherograms. Cucurbitacin A (3 mg in 0·1 ml $CHCl_3$–methanol, 1:1) was applied evenly along a line approximately 13 cm long on a strip of filter paper (Whatman No. 540) 2 cm wide. After evaporation of the solvent enzyme extract (about 4·5 ml) was placed on the line of substrate. The paper was incubated in a moist atmosphere at 30° for 1 hr. After drying, the strip was sewed to a sheet of formamide-impregnated paper and the chromatogram was developed with ethyl acetate–benzene (1:1). The deacetylated product, cucurbitacin N (R_f value approximately 0·05) was well separated in this system from cucurbitacin A (R_f value approximately 0·8; this is higher than the usual value of 0·35–0·40,[1] and is caused by the greater exposure of the impregnated paper to the atmosphere necessitated by the sewing on of the non-impregnated strip). A roughly quantitative assessment of activity in the extract was made by comparing the intensities of the bands of A and N. This method is more rapid and sensitive than procedure (i).

(*c*) *Manometric method.* The technique of Ammon[19] was modified as follows: The substrate (1·74 mmole) was dissolved in ethanol (10 ml) and diluted to 100 ml with water containing 1% Triton X-100 (Rohm and Haas Co.). The enzyme solution, previously adjusted to pH 6·5 (1·5 ml), and $NaHCO_3$ ($2{\cdot}09 \times 10^{-2}$ M, 0·2 ml) were placed in the main compartment of the Warburg flask and the substrate solution (0·3 ml) was added to the side arm. The reaction was allowed to take place at 30° for 30 min. Blanks were run simultaneously to correct for non-enzymic hydrolysis.

Starch Gel Electrophoresis

The method of vertical starch gel electrophoresis of Smithies[20] was used. The gel was prepared with Starch Hydrolysed (Connaught Laboratories, Toronto, Canada) in 0·025 M sodium borate buffer, pH 8·6, and the bridge solution contained 0·3 M sodium borate buffer, pH 8·6. 180 volts were applied across the gel for 17 hr.

[18] F. J. Joubert, *Arch. Biochem. Biophys.* **91**, 11 (1960).
[19] R. Ammon, *Pflügers Arch. Ges. Physiol.* **233**, 486 (1933).
[20] O. Smithies, *Biochem. J.* **71**, 585 (1959).

Staining was carried out using α-naphthyl acetate and tetrazotized *o*-dianisidine (Sigma Chemical Co.),[13] or 6-bromo-2-naphthylcarbonaphthoxy choline iodide and tetrazotized *o*-dianisidine.[21] Cucurbitacin esterases were located by cutting the gel into strips (0·5 or 1 cm wide) which were homogenized with 0·067 M phosphate buffer, pH 6·5, (5 or 10 ml) in a Dounce homogenizer. The extracts were centrifuged for 15 min at 2000 *g* and the activity tested by reaction with cucurbitacin A as described above.

To study the effect of inhibitors the gels were split longitudinally; one half was incubated at 37° for 60 min in 0·25 M phosphate buffer, pH 6·25, containing 10^{-5} M inhibitor before staining; the control half was treated in the same way except that no inhibitor was present in the buffer.

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