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SUBCELLULAR LOCALIZATION OF LIPOXYGENASE AND LIPOLYTIC ACYL HYDROLASE ENZYMES IN PLANTS

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; *Brassica oleracea*; Cruciferae; cauliflower; *Phaseolus multiflorus*; Leguminosae; runner bean; *Pisum sativum*; Leguminosae; pea; lipolytic acyl hydrolase; lipoxygenase; subcellular; linear sucrose gradient.

Abstract—The subcellular localization of two lipid-degrading enzymes, lipolytic acyl hydrolase (LAH) and lipoxygenase (LOX) was studied. In potato tubers the activities of LAH and LOX could not be sedimented, possibly because of the destructive action of these enzymes on subcellular membranes during isolation. In leaves, the enzymes were less active and were obtained mainly in particulate forms but contamination with plastid fractions hindered isolation. In cauliflower florets, the LAH profile on sucrose density gradients was similar to those of other "lysosomal" acid hydrolases, whereas the LOX activity was concentrated in a fraction of high (1·24 g cm^{-3}) isopycnic density, very similar to, but not identical with intact plastids. Microbody, mitochondrial and microsomal fractions had little or no LOX activity. In pea roots, LOX was localized in two separate fractions (isopycnic densities of 1·17 and 1·19 g cm^{-3}) and gave profiles in the gradients coincident with acid phosphatase, but separate from the plastids. The results indicate that LOX is localized in fragile organelles which are separate from mitochondria, microbodies and plastids.

INTRODUCTION

Previous work has shown that potato tubers contain high activities of two lipid-degrading enzymes; a lipolytic acyl hydrolase [1] (LAH) which liberates free fatty acids from the endogenous membrane lipids [2] and a lipoxygenase [3] (LOX) which converts linoleic acid and linolenic acid to their 9-hydroperoxide derivatives. These are the first two steps in a sequence of lipid degrading reactions in potato [4]. The two enzymes were obtained in the particle-free supernatant fraction but it is likely that *in vivo* they are located in discrete but easily ruptured organelles, because disruption of potato tuber tissue causes immediate breakdown of the polar lipids associated with lipoprotein membrane structures within the cell [5].

Because of the high activity of LAH and LOX in potato tubers attempts to prepare subcellular fractions are made difficult by the degradative action of these enzymes on the membrane structures. The LAH enzyme attacks the membrane lipids and the resulting fatty acids not only inhibit the activities of the organelles but also stimulate further breakdown of membrane lipids [2]. Increased destruction may also be caused by the action of LOX in producing hydroperoxides which affect membrane structures [6]. For these reasons it was necessary to select tissues with low but measurable activities of these enzymes to reduce the breakdown of membranes during isolation procedures.

RESULTS

Potato tuber and shoots

Earlier work [7] has shown that tubers of Désirée variety contain relatively low levels of

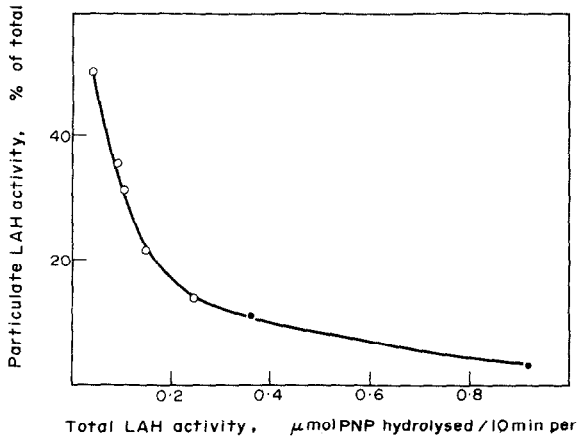

Fig. 1. The relationship between the total LAH activity and the percentage of activity obtained in a particulate form. LAH assay systems (3 ml) contained *p*-nitrophenylpalmitate and Triton X-100 in 0·1 M Pi buffer, pH 7·5 with a homogenate or a particulate fraction obtained by centrifugation at 38000 *g* for 15 min from potato sprouts (○——○) and potato tubers (●——●). Assay by continuous recording spectrophotometric method.

LAH and this variety was therefore chosen for the studies on subcellular localization of enzymes. Most of the LOX and LAH activity was present in the supernatant fraction after differential centrifugation of homogenates prepared from tubers in hypertonic media; only 2·5% of both enzymes remaining in a particulate form. When this experiment was repeated using immature tubers, although little change was observed with LOX, the amount of LAH activity in the particulate fraction increased to 11%. It was also observed that the total LAH activity was much lower in the immature tuber compared with the mature.

Table 1. Localization of LOX and LAH activity in fractions obtained from runner bean leaves by differential centrifugation

Enzyme localization	LOX activity		LAH activity	
	units*	% in homogenate	units*	% in homogenate
1200 *g*, 10 min pellet	7·3	20	0·05	12
4000 *g*, 10 min pellet	8·7	23	0·06	14
38000 *g*, 20 min pellet	9·8	27	0·18	44
105000 *g*, 60 min pellet	2·6	7	0	0
105000 *g*, supernatant	3·1	8	0·14	35

* Enzyme units refer to μmol substrate reacted/10 min/g fr. wt of tissue (O_2 for LOX and *p*-nitrophenylpalmitate for LAH). A runner bean leaf homogenate was centrifuged at the stated *g* and the pellet obtained after each centrifugation was resuspended in the extraction buffer.

Using sprouts of tubers from Désirée and other varieties, the results (Fig. 1) showed an inverse relationship between the total LAH activity and the percentage of activity obtained in a particulate form, and tissue (sprouts and Désirée tubers) with low total LAH activity, yielded 30–50% of the LAH activity in a particulate form. In all these experiments however, most of the LOX activity was in the supernatant fraction.

Differential centrifugation confirmed that the activity of both enzymes in the particulate fraction was localized mainly in the 38000 *g* pellet fraction, with little LAH and no LOX activity associated with the microsomal (100000 *g*) fraction. Solubilization of the particulate enzymes was achieved by the addition of 0·1% Triton X-100. The resulting clear solution retained almost all of the original particulate LAH activity.

Potato and runner bean leaves

Much higher proportions of both enzymes in a particulate form were obtained when leaf material from beans was used but particulate LOX activity could not be demonstrated in potato leaf homogenates. Attempts to separate various organelles by differential centrifugation were not successful, due to contamination, particularly by broken plastids. However, the results in Table 1 show that almost 70% of the activity of both enzymes was recovered in particulate fractions from bean leaves. Again little activity was associated with the microsomal fraction.

Cauliflower florets

Differential centrifugation showed the particulate LOX enzyme to be present mainly in a fraction collected at 1500 *g* in 10 min, whereas most of the particulate LAH was localized in the fraction sedimenting between 1500 and 38000 *g*. About 25–30% of total homogenate activity was recovered in a pellet form for both enzymes. The resolution of these crude fractions was carried out on a discontinuous sucrose gradient. Two particulate fractions were collected from a homogenate prepared from cauliflower florets. The 1500 *g* fraction contained 22% of the LOX activity and 8% LAH activity and the 1500–38000 *g* fraction contained 5% LOX and 28% LAH. They were layered separately on a sucrose density gradient

and centrifuged at 75500 *g* for 3 hr. The particulate LOX from the 1500 *g* pellet was localized in one band (73% recovered) which was held at the interface of the 60% (w/w) layer, with starch grains and debris at the bottom of the tube. The activity in the 1500–38000 *g* pellet was located in a band, which was identified as the mitochondrial fraction using a marker enzyme (succinate dehydrogenase) and which was held at the top of the 45% (w/w) sucrose layer. It remains unclear at the moment whether this small particulate activity is associated with mitochondria or is the result of other organelles adhering to the mitochondria during the purification. LAH activity was located in the mitochondrial band and at the top of the 43% sucrose layer in both separations. Studies on the distribution of various acid hydrolases showed strong activity from these two bands. Further work is necessary to prove that the organelle containing the acyl hydrolase is "lysosomal", but the two lipid degrading enzymes are probably located in quite different subcellular organelles in cauliflower florets. From the evidence of marker enzymes, the band containing LOX activity did not contain mitochondria or "lysosomes" and this was in agreement with the electron micrographs. These did however, show the presence of plastids and other bodies and as the results demonstrated organelles held at the interface of the gradients, further purification was carried out using linear gradients. In preliminary experiments a homogenate was layered directly on a linear sucrose gradient to avoid the disruption which occurs during the initial centrifugation and resuspension of the pellet (Dr. H. Beevers, a personal communication). However, the LAH could not be separated from the LOX quickly without the initial centrifugation and the evidence from the earlier results suggested that the longer the two enzymes remained in contact, the higher the loss of particulate activity would be. In fact very little LOX activity was recovered in fractions collected from a gradient when a homogenate was layered.

The results of a linear gradient using a 1500 *g* pellet showed that the LOX activity was located at a density of 1·24 g cm^{-3} (51·5% w/w sucrose), well separated from the microbodies which were located by the catalase marker enzyme and gave a peak at 1·22 with a shoulder at 1·20 g cm^{-3}. Malate dehydrogenase, a known peroxisome and glyoxysome marker enzyme [8], followed a similar pattern to that observed with catalase. This enzyme has been found in all plant peroxisomes isolated and it can be assumed that cauliflower peroxisomes would contain a malate dehydrogenase isoenzyme. Huang and Beevers [9] reported that the catalase peak for microbodies, isolated from cauliflowers, had an isopycnic density of 1·22 and for mitochondria 1·18 g cm^{-3}. The small quantity of mitochondria present in the 1500 *g* pellet also had an isopycnic density of 1·18. The other bodies located in this area of high sucrose concentration are plastids which have been reported in castor bean endosperm [10], sunflower cotyledons [11] and spinach leaves [12], to have an isopycnic density between those of the microbodies and the mitochondria. With pea roots [13], however, intact plastids have a higher density (1·23) than microbodies (1·19). The enzyme chosen as the plastid marker was triose phosphate isomerase [11,13]. The peak of intact plastids coincided with LOX activity. A technique has been developed by Miflin and Beevers [14] for the isolation of intact pure plastids which involves a brief centrifugation of homogenates on density gradients, when plastids quickly reach their equilibrium density and microbodies and mitochondria barely move into the gradient. This method was employed using a 1500 *g* pellet but it was found necessary to remove debris before layering the resuspended pellet by centrifuging at 290 *g* for 2 min. If this was not carried out there was a tendency for some debris to cling to the walls of the tube and to be collected with the organelles.

The result of a rapid gradient separation (10 min at 27000 *g*) are shown in Fig. 2a where the plastids have almost reached their equilibration density (1·22 g cm^{-3}) and coincident with this band is a band of LOX activity. Microbodies and mitochondria have barely moved into the gradient, the former having a density of 1·14 g cm^{-3}.

From these results one would presume that LOX resides in the plastids. However, evidence from electronmicrographs was not so convincing. Fractions taken from the catalase and succinate dehydrogenase bands, after fixing and embedding, showed predominantly microbodies and mito-

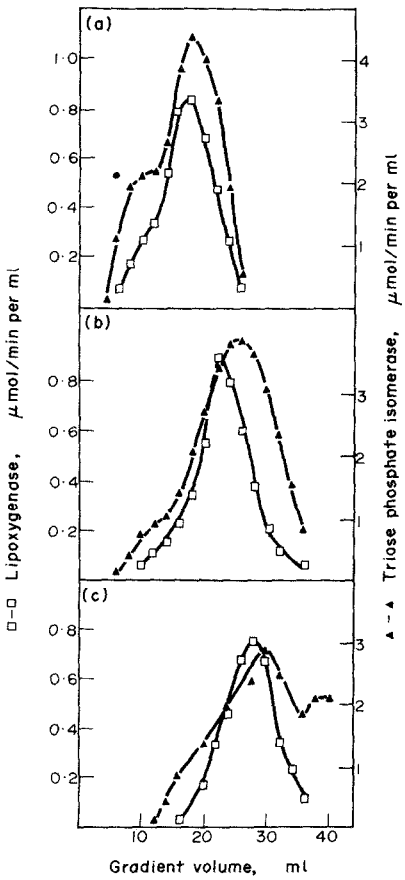

Fig. 2. Distribution of LOX and triose phosphate isomerase on a linear sucrose gradient of a 1500 *g* particulate fraction from cauliflower florets. The resuspended pellet was centrifuged at 290 *g* for 2 min before being layered on the gradient. The tubes were centrifuged at 2a, 27000 *g*, for 10 min; 2b, 12000 *g* for 7 min; 2c, 12000 *g* for 5 min.

chondria, but fractions from the triose-P isomerase band showed plastids, a few mitochondria and some smaller spherosome-like osmiophilic bodies.

It was therefore decided to attempt an even slower speed and time for the gradient separation to show whether the triose-P isomerase and LOX enzymes were located in one or two separate organelles. Results of these experiments are shown in Fig. 2b and 2c where centrifugation conditions were 12000 *g* for 7 min for the former and 5 min for the latter. It can be seen in Fig. 2b, where the bodies are in the middle of the gradient, that there are 2 separate bands, with LOX at a density of 1·18 g cm^{-3} and triose-P isomerase at 1·165 g cm^{-3}.

Further confirmation was obtained by means of differential centrifugation on the distribution of four marker enzymes in a particulate form (Table 2). They upheld the previous results that LOX does not reside in the microbodies or mitochondria as both marker enzymes increased noticeably in the 10800 *g* pellet, whereas LOX showed only a slight increase. The marker enzyme for plastids did not increase significantly after the first centrifugation whereas LOX almost doubled in activity, this giving further proof that 2 organelles are involved. The difference in the final percentages of particulate activity cannot be used to further the argument, because in an analogous case [13] in spinach leaves a greater proportion of nitrate reductase (13%) than of the triose-P isomerase (8%) was associated with intact plastids due to the presence of a non-particulate isoenzyme of triose-P isomerase in addition to the plastid isoenzyme.

Pea roots

Experiments showed that the amount of LOX recovered in a particulate form was lower than with cauliflowers. The particulate activity was

Table 2. Localization of enzymes in fractions obtained from cauliflower florets by differential centrifugation

Enzyme localization	Lipoxygenase activity		Triose-P isomerase activity		Catalase activity		Succinate dehydrogenase activity	
	units*	% in homogenate	units*	% in homogenate	units*	% in homogenate	units*	% in homogenate
Homogenate	2·0		26·5		69		52	
750 *g* pellet	0·18	9	2·2	8·5	4·6	6·5	4·7	9
1500 *g* pellet	0·3	15	2·5	9·0	10·6	15·5	11·6	22
10800 *g* pellet	0·36	18	2·7	9·5	29·5	43	21	40

* Enzyme units refer to μmol substrate reacted/10 min/g fr. wt of tissue. Three cauliflower floret homogenates were centrifuged at the stated *g* for 10 min and the pellets resuspended in the extraction buffer.

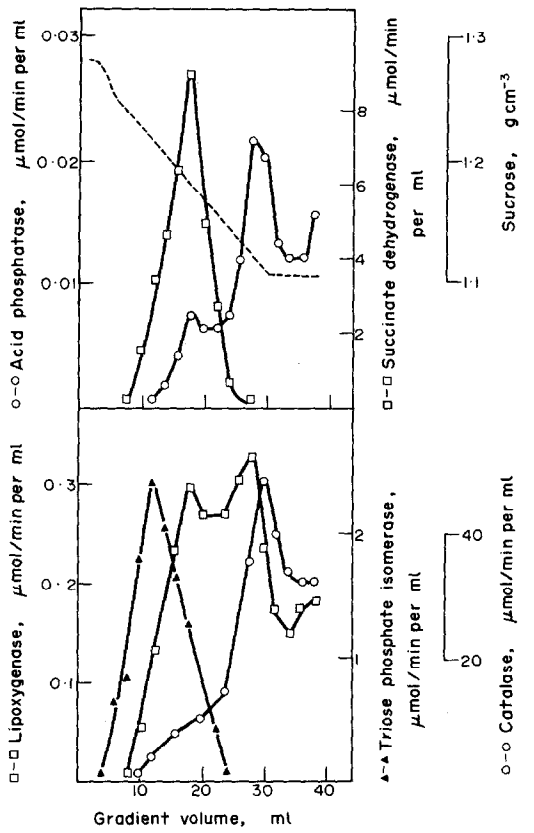

Fig. 3. Distribution of enzymes on a linear sucrose gradient of a 38000 *g* particulate fraction isolated from pea roots. The gradient was centrifuged for 15 min at 27000 *g*.

also not confined to the 1500 *g* fraction but was equally divided between this fraction and the 38000 *g* pellet. Plastids on the other hand had a higher activity in the particulate (15%) than previously found from the cauliflower florets.

It has been reported [15] that LOX activity is high in young pea seedlings and then falls to a minimum after 7–8 days germination. We have also found in the roots a fall in activity over the first 9 days where 3 day roots contained 17 μmol/min/g of LOX activity and after 9 days germination only 6·7 μmol. This fall is not related to a concentration of activity in the root tip, since results on comparing activity on the terminal 3 cm of a root with 6 cm of the remaining tissue, taken from a 7 day root, showed on a fr. wt basis, lower activity in the terminal portion. It was also found that higher recoveries of the particulate LOX enzyme occurred if small quantities of tissue were extracted at one time. Thus, less than 10 g of 3–5 day old pea roots yielded 10% of the LOX activity in a particulate form.

Several linear sucrose density gradient separations were effected using a 38000 *g* particulate fraction and homogenates where a rapid gradient separation prevented large losses. With the brief centrifugation technique, both LOX and acid phosphatase were located over a wide area of the gradient (Fig. 3), with the main peak of activity at a density of 1·12 g cm^{-3}, slightly ahead of the microbodies. The heavier particles displaying activity around 1·18–1·19 g cm^{-3} are coincident with the mitochondria with the plastids further ahead at 1·22 g cm^{-3}. When the centrifugation was decreased to 10 min at 12000 *g*, LOX and acid phosphatase activity were still observed around 1·19 cm^{-3} with the main band at 1·11, whereas the mitochondria had not moved very far into the gradient (1·12 g cm^{-3}). Therefore the only organelles capable of trapping LOX, if this is the reason for the two peaks, were plastids, which were again further ahead in the gradient at 1·21 g cm^{-3}. It should be noted that with the same brief centrifugation technique on cauliflower particulates, the sole LOX band was always sharply defined. The result of an isopycnic gradient centrifugation of pea roots showed that triose-P isomerase and cytochrome oxidase are separated, at a density of 1·23 and 1·20 g cm^{-3} respectively, and the LOX activity coincides with acid phosphatase and catalase at 1·19. The lighter peak of LOX and acid phosphatase activity, band together at a density of 1·17 g cm^{-3}. Both bands of LOX and acid phosphatase are much sharper as a result of isopycnic gradient centrifugation and it would appear that the activity found in the centre of the brief centrifugation gradient equilibrates towards the heavier peak.

Properties of the linoleate-oxidising fractions

The particulate fraction isolated from cauliflower florets after a 1500 *g* centrifugation, oxidised linoleic but not oleic acid. It was heat labile and not effected by KCN (10 mM) at a pH of 5·5. There was also a linear relation between enzyme concentration and reaction rate.

A homogenate or a 1500 *g* particulate fraction lost activity very quickly; 80% in 24 hr at +1°.

However, fractions collected from sucrose gradients retained their activity for much longer periods. When homogenates were prepared from an extraction buffer containing 40% sucrose, their activity remained high and a particulate fraction isolated from these homogenates also retained activity for several days.

Fractions of pea root tissue from a homogenate and from both linoleate-oxidising bands, isolated from a sucrose gradient at equilibrium density, were incapable of oxidising oleate, moreover they showed no activity after boiling with linoleic acid as substrate. With 10 mM KCN present in the assay system, no inhibition occurred, provided the pH was kept at 6·2, the optimum pH for pea root LOX.

DISCUSSION

From the results with pea roots reported in this paper the LOX activity in the sucrose gradient coincides with acid phosphatase, previously located in "lysosomes" [16], in two separate peaks. In no instance is there any indication that plastids contain any LOX activity and from the results of both the brief centrifugation technique and the isopycnic gradient centrifugation it does not appear that mitochondria or microbodies contain any activity.

With cauliflower floret extracts however, only one peak of LOX activity was observed in the gradient and this was not coincident with acid hydrolase activity or any other of the marker enzymes tested.

Previously it has been reported that LOX activity is associated with microbodies in oat and wheat leaves [17], in mitochondria and chloroplasts in alfalfa leaves [18], low activity in chloroplasts and high in etiolated plastids from pea leaves [19], and in peroxisome like bodies and mitochondria in pea seedlings [19]. From the results in this work it appears that confusion can easily arise if only one technique is employed in separating various organelles.

In pea roots the LOX activity has a similar distribution to acid hydrolase enzymes, whereas in the cauliflower florets it is quite separate from the "lysosomal" enzymes. We have so far been unsuccessful in isolating a fraction which is homogeneous in electron micrographs. It is not yet clear whether the LOX-containing bands in sucrose density gradients represent intact organelles or whether the behaviour of these bodies in the gradients is affected by uptake of sucrose which is known to penetrate into "lysosomes" [20] and other organelles [21].

The other lipid degrading enzyme, LAH, from cauliflower florets is located in the sucrose gradients at a lower isopycnic density than LOX and coincides with other acid hydrolases. The location of the enzyme in the gradient from pea roots has not yet been confirmed but the presence of two bands of acid phosphatase activity has been observed. These bands appear similar to the light and heavy "lysosome" fractions isolated in potato shoots by Pitt and Galpin [16].

EXPERIMENTAL

Plant materials. Cauliflower (*Brassica oleracea*), potato tubers (cv Désirée) (*Solanum tuberosum*), and runner bean leaves (*Phaseolus multiflorus*) were grown at the Institute or obtained from local stores. Pea seedlings (*Pisum sativum* cv Alaska) were grown in moist vermiculite at room temp. Cauliflowers, when freshly harvested, were held at 5° for 2 days before use. The top 5 mm of inflorescences were used. Sprouts 10 cm in length, with tips and leaf removed, were taken from tubers stored in the dark at 10°. Leaves and roots were rinsed in H_2O, blotted and held at 5° for 1 hr before use. All material was cut into small pieces directly into H_2O at 0°.

Tissue extraction. Grinding material contained 0·3 M mannitol, 1 mM EDTA, 10 mM $MgCl_2$, 4 mM cysteine, 1% bovine serum albumin (BSA) and 0·1 M HEPES buffer, pH 7·4. With potato tubers, potato sprouts and pea roots 0·2 mM 2-mercaptobenzothiazol was added to the medium. With pea roots 25% w/w sucrose replaced mannitol. Plant materials, after being shredded with razor blades in the cold medium, were gently ground with sand in a pestle and mortar. The homogenate was passed through several layers of muslin and then centrifuged at either 1500 *g*, or 10800 *g* for 10 min, or 38000 *g* for 15 min. The crude particulate pellet was resuspended in a few ml of the original grinding medium. Particulates were used directly, or layered on a discontinuous or linear sucrose gradient.

Density gradients. These were w/w sucrose solns made up to 100% with 0·1 M HEPES buffer, pH 7·4, containing 1 mM EDTA and 0·2% BSA. Discontinuous gradients were composed of 10 ml of a 60% sucrose cushion, 15 ml 45%, 15 ml 43% and 10 ml of a 25% sucrose soln. Continuous gradients with cauliflower extracts for long centrifuge runs were 8 ml of 55% sucrose, followed by 36 ml of a linear gradient from 55 to 33% with a final 6 ml of 33%. For brief runs the gradient was composed of 10 ml 60% followed by 30 ml from 55 to 30% with a final 10 ml of 30%. The gradient used for pea roots consisted of 5 ml 60%, a linear gradient of 30 ml 55 to 25%, followed by 10 ml 25% sucrose. When pea root homogenates were used 12 ml were layered on to only 5 ml 25% sucrose, otherwise around 4 ml particulate fraction were layered and the tubes placed in a SW 25-2 rotor in a Beckman Spinco L2 centrifuge and centrifuged for either 3 hr at

25000 rpm (75500 *g*) or 7–15 min at either 10000 rpm (12000 *g*) or 15000 rpm. The rotor was allowed to decelerate without the brake for the shorter runs. Separation into 2 ml fractions was accomplished with the aid of a pump and fraction collector by taking samples from the bottom of the tube. Sucrose concentrations of the individual samples were determined refractometrically.

Enzyme assays. Catalase was determined by the method of Luck [22]. Triose phosphate isomerase was coupled with glycerol phosphate dehydrogenase and followed by the oxidation of NADH [23], and malate dehydrogenase [24] was also assayed by the oxidation of NADH. Succinate dehydrogenase was determined by the method of Singer *et al.* [25] and cytochrome oxidase by Tolbert *et al.* [26]. Lipoxygenase was assayed by a polarographic method [7], using ammonium linoleate as substrate and acetate buffer, pH 6·2 for pea root tissue and pH 5·5 for all other tissues. Lipolytic acyl hydrolase activity was determined using *p*-nitrophenylpalmitate as substrate either by a continuous recording spectrophotometric method [1] or by incubation for measured times and subsequent determination of the *p*-nitrophenol released [1]. Acid hydrolase activity was determined by assaying for acid phosphatase, phosphodiesterase and ribonuclease by the method of ref. [27] and carboxylic esterase by the method of ref. [28], modified by ref. [29].

Electron microscopy. Pooled fractions from sucrose gradients were fixed with 2·5% glutaraldehyde overnight. The suspension was then dil with an equal vol of 35% sucrose in gradient buffer and was centrifuged for 45 min at 38000 *g*. The pellet was washed once with 0·4 M sucrose in Palades buffer, pH 7·4 and recentrifuged, then fixed in an agar block and cut sections were fixed for 1½ hr with 1% OsO_4 in Palades buffer, pH 7·4, washed and then dehydrated in an alcohol series, followed by propylene oxide and embedded in Epon. Silver–Gold sections were cut with glass knives and mounted on 100 mesh Formvar coated copper grids.

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REFERENCES

1. Galliard, T. (1971) *Biochem. J.* **121**, 379.
2. Galliard, T. (1971) *European J. Biochem.* **21**, 90.
3. Galliard, T. and Phillips, D. R. (1971) *Biochem. J.* **124**, 431.
4. Galliard, T. (1975) in *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (Galliard, T. and Mercer, E. I., eds.). pp. 319–356. Academic Press, London.
5. Galliard, T. (1970) *Phytochemistry* **9**, 1725.
6. Christophersen, B. O. (1968) *Biochem. Biophys. Acta.* **164**, 35.
7. Galliard, T. and Matthew, J. A. (1973) *J. Sci. Food Agr.* **24**, 623.
8. Schnarrenberger, C., Oeser, A. and Tolbert, N. E. (1971) *Plant Physiol.* **48**, 566.
9. Huang, A. H. C. and Beevers, H. (1971) *Plant Physiol.* **48**, 637.
10. Cooper, T. G. and Beevers, H. (1969) *J. Biol. Chem.* **244**, 3507.
11. Schnarrenberger, C., Oeser, A. and Tolbert, N. E. (1972) *Plant Physiol.* **50**, 55.
12. Dalling, M. J., Tolbert, N. E. and Hageman, R. H. (1972) *Biochem. Biophys. Acta.* **288**, 505.
13. Miflin, B. J. (1974) *Plant Physiol.* **54**, 550.
14. Miflin, B. J. and Beevers, H. (1974) *Plant Physiol.* **53**, 870.
15. Anstis, P. J. P. and Friend, J. (1974) *Planta (Berlin)* **115**, 329.
16. Pitt, D. and Galpin, M. (1973) *Planta (Berlin)* **109**, 233.
17. Gardner, D. C. J. and Friend, J. (1974) (Personal communication).
18. Grossman, S., Ben-Aziz, A., Ascarelli, I. and Budowski, P. (1972) *Phytochemistry* **11**, 509.
19. Haydar, M. and Hadziyev, D. (1973) *J. Sci. Food Agr.* **24**, 1039.
20. Beaufay, H. (1969) in *Frontiers of Biology* 14*B*, *Lysosomes in Biology and Pathology*, Vol. 2, 515 (Dingle, J. T. and Fell, H. B., eds.). North Holland.
21. Cohen, E., Shain, Y., Ben-Shaul, Y. and Meyer, A. M. (1971) *Can. J. Botany* **49**, 2053.
22. Luck, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. ed.) p. 885, Academic Press, New York.
23. Beisenkerz, G. (1955) in *Methods in Enzymology* (Colowich, S. P. and Kaplan, N. O., eds.), Vol. 1, p. 387, Academic Press, New York.
24. Tolbert, N. E., Oeser, A., Yamazaki, R. K., Hageman, R. H. and Kisaki, T. (1969) *Plant Physiol.* **44**, 135.
25. Singer, T. P., Oestreicher, G. and Hogue, P. (1973) *Plant Physiol.* **52**, 616.
26. Tolbert, N. E., Oeser, A., Kisaki, T., Hageman, R. H. and Yamazaki, R. K. (1968) *J. Biol. Chem.* **243**, 5179.
27. Pitt, D. and Galpin, M. (1971) *Planta (Berlin)* **101**, 317.
28. Nachlas, M. M. and Seligman, A. M. (1949) *J. Biol. Chem.* **181**, 343.
29. Pitt, D. and Coombes, C. (1969) *J. Gen. Microbiol.* **56**, 321.