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FURTHER STUDIES ON THE SUBCELLULAR LOCALIZATION OF LIPID-DEGRADING ENZYMES

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; *Brassica oleracea*; Cruciferae; cauliflower; calabrese; *Pisum sativum*; Leguminosae; pea; lipolytic acyl hydrolase; lipoxygenase; subcellular; linear sucrose and Ficoll gradients.

Abstract—Further work on the subcellular localization of two lipid-degrading enzymes, lipolytic acyl hydrolase (LAH) and lipoxygenase (LOX) has been carried out on brassica florets, potato shoots and pea roots. In all cases, the LAH profile on sucrose and Ficoll density gradients was coincident with 'lysosomal' acid phosphatase activity. However, the localization of LOX activity was different for each tissue. In pea roots the activity of LOX was localized in the 'lysosomal' fraction, whereas with brassica florets (cauliflower and calabrese) it was present in a heavy body with a similar density to plastids and in potato shoots LOX gave only low particulate recoveries.

INTRODUCTION

Plant tissues contain active enzymes which, during the isolation of subcellular organelles, may attack the lipids of the limiting membrane of the organelles. One of these, lipolytic acyl hydrolase [1] (LAH), liberates free fatty acids from the endogenous membrane lipids. Previous work [2] showed that the localization of this enzyme in cauliflower florets was similar to those of other 'lysosomal' acid hydrolases. Another enzyme, lipoxygenase [1] (LOX), converts the unsaturated fatty acids, linoleic and linolenic acid to their hydroperoxide derivatives. In cauliflower floret tissue this enzyme was concentrated in a fraction of high isopycnic density on a sucrose gradient, whereas in pea roots it banded in the gradient with acid phosphatase [2] (AP).

The present paper describes further work, using particulate fractions isolated from brassica florets, potato shoots and pea roots on Ficoll and sucrose gradients, with and without Mg^{++} ions, in order to clarify the location of the two enzymes.

RESULTS

Cauliflower florets

LAH activity. The previous work [2], on the differential centrifugation of cauliflower homogenates was repeated, with the omission of $MgCl_2$ from the buffer, since it is known that Mg^{2+} ions produce aggregation of organelles [3], but again the majority of the LAH particulate activity (22% of the homogenate activity) was localised in the 1500–38 000 g pellet and the LOX in the 1500 *g* pellet.

The resolution of the crude 1500–38 000 *g* pellet was carried out on a linear sucrose gradient. The particulate LAH activity was coincident with AP activity in two separate peaks at a density of 1.16 and 1.08 g cm^{-3} (Fig. 1a). The latter band was visible near the top of the gradient. Microbodies (marker enzyme, catalase) and mitochondria (cytochrome oxidase) equilibrated at 1.22 and 1.18 *g* cm^{-3} respectively (Fig. 1b) which confirmed the earlier results [2] when a 1500 *g* pellet was layered. It was noted in the latest experiments where the pellet contained a higher proportion of mitochondria, that more contamination from microbodies occurred in the mitochondrial band on the gradient than when a 1500 *g* pellet was used. The majority of the plastids (triose phosphate isomerase) and organelles containing LOX activity sedimented at 1500 *g*. Those still present in the 1500 to 38 000 *g* fraction were localized near the bottom of the sucrose gradient at 1.24 g cm^{-3}.

From the results of linear sucrose gradients, acyl hydrolase activity was coincident with AP activity and the previous results [2] also confirmed that LAH banded with several acid hydrolases. However, from their density on the gradients, the enzymes could well be coincident with the microsomal fraction which is known to band in this area [4]. The marker enzyme NADH–cytochrome *c* reductase was used to identify the microsomal fraction which had an isopycnic density of 1.13 g cm^{-3} and a shoulder at 1.16, the latter having the same density as the hydrolase activity (Fig. 1a). It is known [4] that when Mg^{2+} ions are included in the sucrose gradients to prevent the dissociation of membrane-bound ribosomes during the centrifugation, the isopycnic density of the membrane fraction is increased. The experiments were therefore repeated with 3 mM $MgCl_2$ in the gradients The results showed no change in the two densities of LAH and AP (1.16 and 1.08 g cm^{-3}), but the microsomal fraction had moved further down the gradient at 1.17 g cm^{-3}. The broad spread of the microsomal fraction on gradients containing Mg^{2+} ions which has been observed previously [5] and is noted below in the section on pea roots, did not occur with the floret tissue. This may be due to the fact that with cauliflower florets a pellet was layered on the gradient containing Mg^{2+} ions whereas an homogenate was used in the other experiments.

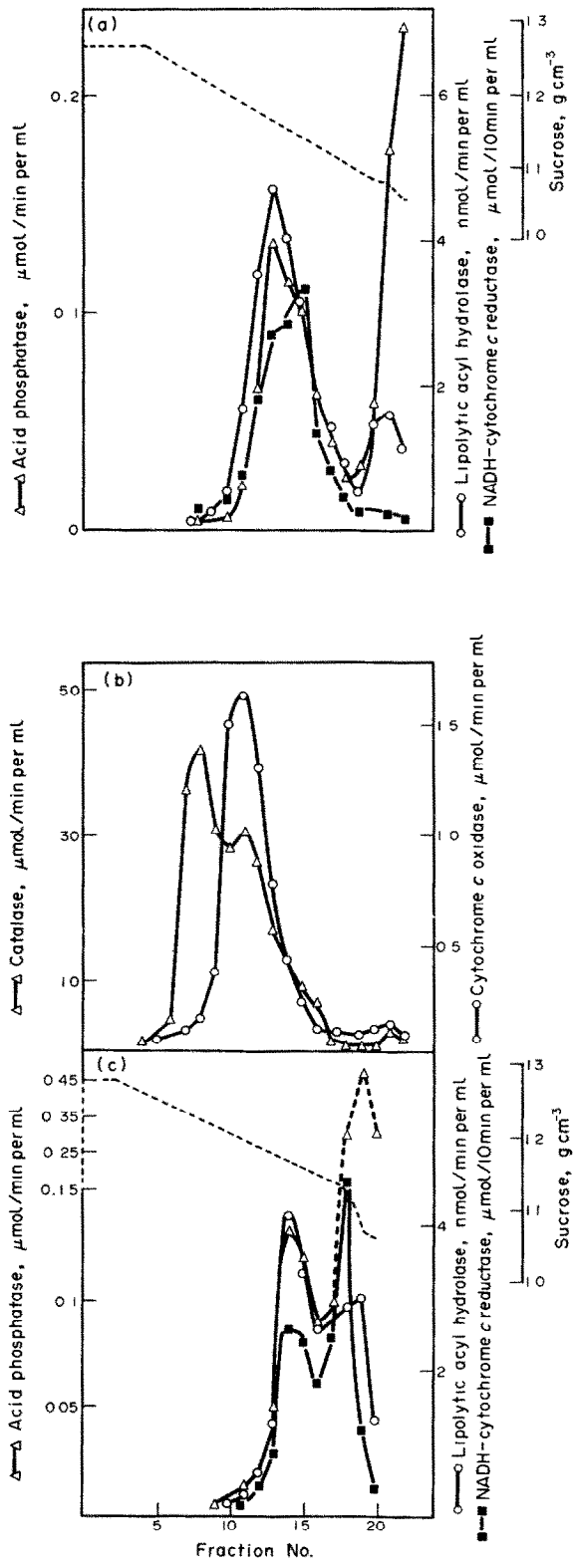

Fig. 1. Distribution of enzymes on a linear sucrose gradient of a 1500–38000 *g* particulate fraction isolated from cauliflower florets. The gradients were centrifuged for 3 hr at 75500 *g*. (a and b) linear sucrose from 58–16% with a final 5 ml of 16%. (c) linear sucrose from 60–30% with a final 5 ml of 30%.

In both these experiments, however, the peak of LAH and AP activity at 1.16 g cm^{-3} contained a large amount of NADH–cytochrome *c* reductase and it was impossible to determine whether any of the latter activity was associated with the 'lysosomal' peak. A further gradient was therefore constructed with a linear sucrose concentration from 60% w/w to 30% with a final 5 ml of 30% sucrose and no Mg^{2+} in the gradient. The results, (Fig. 1c) showed a clear resolution between LAH activity (1.16 g cm^{-3}) and NADH–cytochrome *c* reductase (1.12 g cm^{-3}). A further small peak of NADH–cytochrome *c* reductase activity was also coincident with LAH and AP. This enzyme activity could be associated with contaminating organelles or with the membranes of 'lysosomes'. No increase in activity was observed when antimycin-A was omitted from the assay and therefore mitochondrial activity is not responsible [6] and it appears unlikely that microbodies, isopycnic density 1.22, would produce the pattern of activity recorded. It is not known whether a contaminating microsomal fraction or lysosomal activity was responsible, but it is worth noting that the membranes of animal lysosomes contain NADH–cytochrome *c* reductase [6].

The particulate fraction contained 22% of the homogenate LAH activity and 75% of the activity in this fraction was recovered on sucrose gradients in the 1.16 g cm^{-3} band with the remainder in the 1.08 g cm^{-3} band. The percentage of AP activity in a particulate form however was poor, around 4%, and it appeared that the majority was localized in the 1.08 band. However, when the last five fractions of the gradient were combined, centrifuged and the pellet resuspended, 88% of the LAH activity was in the pellet compared with only 35% of the AP activity. This means that either further breakdown of the particulate AP had occurred after preparation of the pellet or that the unwashed pellet contained a high concentration of soluble activity. The amount of particulate activity of the other organelles in the 1500–38000 *g* pellet was relatively high, with cytochrome oxidase at 80%, catalase 32%, isocitrate lyase 17% and NADH–hydroxy-pyruvate reductase 35%.

It is known [7] that microbodies may readily lose some of their enzymes to the surrounding medium, whereas others such as catalase, are not lost. This may explain the difference between the percentage recovery of the two enzymes, LAH and AP, if they are contained in the same organelle, especially if the organelle has a wide variation of physical properties such as a 'lysosome' or vacuole. Large vacuoles inevitably burst during preparation [8] and only the small vacuoles in meristematic cells are not destroyed during the grinding of the tissue. When pea roots and potato shoots were used, high AP activity was recovered in the particulate fractions and with the latter tissue the white distal end of the shoot gave a higher particulate recovery for both enzymes than when older tissue was extracted. It was also found that when the tips of floret inflorescences were ground in place of the usual 5 mm pieces there was an increase in activity for both enzymes in the particulate fraction, LAH giving a 30% recovery and AP 6%. Pitt and Galpin [9] reported that 'lysosomes' from potato shoots and leaves possessed AP-binding sites that involved phosphatide acyl ester linkages. As stated above no difficulty was experienced in the recovery of particulates containing AP from potato shoots but only from the cauliflower florets. No increase in recovery was observed when the buffer used by Pitt,

2.5% Ficoll and 8.5% sucrose, replaced mannitol in the extraction of the florets. A high proportion of the AP activity was again observed in the soluble fraction after the particulate had been layered on a Ficoll gradient and a comparison between the levels of particulate and soluble AP activities on the three types of gradient, 16% sucrose, 30% sucrose and Ficoll and 8.5% sucrose, showed that all contained a high amount of soluble AP activity at the end of a run. LAH and AP had only one peak of activity in the Ficoll gradient at 1.08, mitochondria were at 1.13 and microbodies at 1.10 g cm^{-3}. The activity peak of NADH–cytochrome *c* reductase was at 1.07 with a further peak at 1.08, coincident with LAH and AP.

Damage can well occur to fragile organelles during differential centrifugation and resuspension in buffer [3]. Therefore it may be advantageous to use a cushion of sucrose solution, in a similar manner to that employed during sucrose gradient experiments. This procedure was tried using 5 ml of 50% w/w sucrose in buffer at the bottom of the tube and slowly adding 20 ml of homogenate, prepared from the usual sized florets. After centrifuging at 38 000 *g* for 10 min the organelles could be seen at the top of the cushion. The organelles together with some of the sucrose layer were removed from the tube. Particulate activity of LAH was 30% and AP 13% after corrections had been made for any soluble enzymes present in the fraction. The organelles collected by this method are more heavily contaminated by the soluble fraction than the more conventional way. However, it may be useful in concentrating a homogenate without causing too much loss.

Pea roots

LAH and LOX activities. Previous work [2] on the separation of organelles from pea roots was carried out on sucrose gradients. It is known that some bodies are effected by the uptake of sucrose [10, 11] and therefore further experiments were undertaken using Ficoll gradients to confirm the location of LOX and to identify the organelle containing LAH activity. Table 1 shows the isopycnic densities of various organelles from a 38 000 *g* resuspended pellet in both sucrose and Ficoll gradients. The two separate peaks observed in the sucrose gradients for both LOX and AP activity do not occur when Ficoll is used. We reported in the previous paper [2] that LOX activity bands coincidently with AP and this is confirmed from these results which also show that the hydrolase activity is in the same band. They also indicate that the mitochondria do not contain any LOX activity (sucrose and Ficoll isopycnic centrifugation) nor do the microbodies (sucrose, brief centrifugation and Ficoll, isopycnic). Plastids, which in the sucrose gradient were heavier than the other organelles and gave a sharp band, were not so well resolved with Ficoll and often were

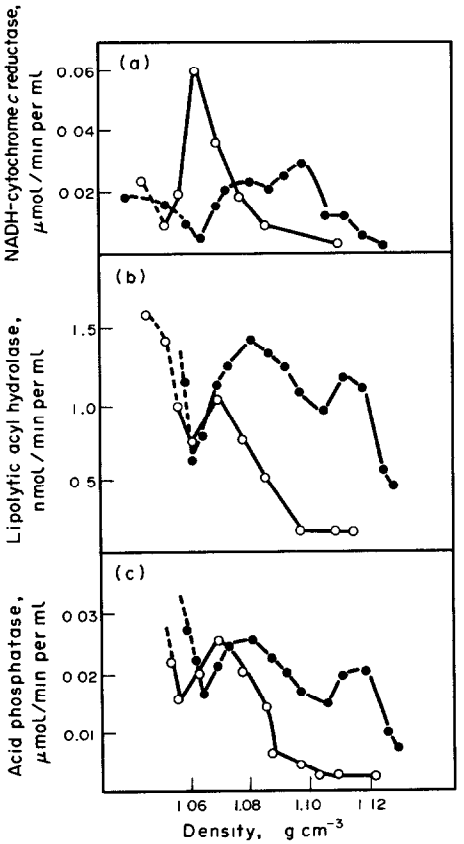

Fig. 2. The effect of the inclusion of magnesium chloride (3 mM) in the linear Ficoll gradient on the sedimentation behaviour of (a) NADH–cytochrome *c* reductase, (b) lipolytic acyl hydrolase and (c) acid phosphatase. The gradients containing pea root homogenate were centrifuged for 2.5 hr at 75 500 *g*. ●—●, centrifugation on gradient with magnesium chloride. ○—○, centrifugation on gradient without magnesium chloride. The dotted line shows supernatant fraction.

Table 1. Isopycnic densities of organelles isolated from pea roots in sucrose and Ficoll gradients

Organelle	Marker enzyme	Density in sucrose (g cm^{-3})	Density in Ficoll (g cm^{-3})
Plastids	Triose phosphate isomerase	1.23	1.18+
Mitochondria	Cytochrome *c* oxidase	1.20	1.11
Microbodies	Catalase	1.19	1.085
'Lysosomes'	Acid phosphatase	1.19/1.17	1.07
	Lipoxygenase	1.19/1.17	1.07
	Lipolytic acyl hydrolase	—	1.07
Microsomal fraction	NADH–cytochrome *c* reductase	—	1.065

A 38 000 *g* particulate fraction was layered on a linear sucrose or Ficoll gradient and centrifuged for 2.5 hr at 75 000 *g*.

identified at the bottom of the tube. To overcome this, a cushion of 50% w/w sucrose was placed at the bottom of the Ficoll gradient and at the completion of the run plastids were identified on the surface of the sucrose at a density of 1.18 g cm^{-3}. The band was contaminated with other organelles and it would appear that Ficoll is not as suitable as sucrose for use with plastids. The location of the microsomal fraction was slightly below the LAH, LOX and AP activities at a density of 1.065 g cm^{-3}.

The effect of Mg^{2+} ions in the Ficoll gradient on the distribution of the enzymes when an homogenate was layered, is shown in Fig. 2. In the presence of Mg^{2+} ions the activity of NADH–cytochrome *c* reductase (Fig. 2a) at 1.065 g cm^{-3} was lost and was spread broadly around 1.10 g cm^{-3}, whereas LAH and AP activities (Fig. 2b and 2c) were slightly increased from 1.07 to 1.08 g cm^{-3}. A further peak of activity of these two enzymes was apparent at 1.11 g cm^{-3}, when Mg^{2+} ions were present and may be due to organelles adhering to the mitochondria which had the same isopycnic density (1.11 g cm^{-3}) in both experiments. As with the work on the cauliflower florets, some of the NADH–cytochrome *c* reductase activity may well be associated with the organelle containing the LAH and AP activities both with and without Mg^{2+} ions in the gradient.

The particulate fraction layered on the Ficoll gradient contained 21% of the homogenate LAH activity and 10% AP activity and when an homogenate was applied, 40% of the LAH and 9% of the AP activity were recovered from the gradient at a density of 1.07 g cm^{-3}.

Potato shoots

LAH activity. Initial experiments were carried out on an homogenate prepared from potato shoots applied to a linear Ficoll gradient. Both LAH and AP had only one peak in the gradient at 1.06 g cm^{-3}. Mitochondria equilibrated at a density of 1.115, microbodies remained close to the soluble fraction at the top of the gradient at 1.04 g cm^{-3} and plastids were at the bottom, 1.12+. Isocitrate lyase, identified by Pitt [9] at a density of 1.14 g cm^{-3} could not be found, nor the heavier AP peak at 1.10 g cm^{-3}. Recovery of both LAH and AP activities from the gradients was between 20–40% of the original homogenate.

Differential centrifugation experiments were carried out before a particulate fraction was used, in order to find the most active fraction and whether Mg^{2+} ions had any effect on the sedimentation. Table 2 shows that even in the presence of Mg^{2+} ions in the extraction buffer, considerable activity was recovered at 105 000 *g*. The results, with a resuspended pellet from a 78 000 *g* centrifugation layered on a linear Ficoll gradient, showed LAH and AP activities coincident in a single peak at 1.06 g cm^{-3}. A much lower recovery of LAH from the gradient was obtained from a pellet (9.5%) than when an homogenate was layered.

Further experiments were carried out using a homogenate and a linear sorbitol gradient, considered to be superior to sucrose [12]. The peak of AP and LAH activity was at a density of 1.09 g cm^{-3} and 30% of the homogenate activity was recovered in this band. A small amount of activity of both enzymes (3%) was also found at 1.19 g cm^{-3} but was coincident with catalase. Mitochondria equilibrated at a density of 1.08 and plastids were further ahead on the gradient at 1.22+ g cm^{-3}.

LOX activity. Homogenates, prepared from Désirée, Majestic and Golden Wonder potato shoots, after centrifugation at 80 000 *g*, contained around 4% of their LOX activity in a particulate form. This amount of activity was found to be insufficient to give meaningful results on sucrose gradients and therefore variations in the extraction buffer and grinding techniques were attempted. Compared to the normal pH 7.4 buffer, both lower, pH 6.2 and higher, pH 8.4 buffers were tried with no increase in particulate recovery. 0.3 M mannitol, 2.5% Ficoll and 5% Dextran gave slightly higher recoveries than 0.25 M sucrose, Ficoll and Dextran [13] or 0.5 M sorbitol and Ficoll. It is known that the content of specific enzymes in microbidies may vary during the isolation of organelles [7]. However, no changes were observed by replacing HEPES buffer by Tris–HCl buffer or by varying the ratio of medium to tissue. The omission of $MgCl_2$, EDTA or BSA also had no effect. Changes in the extraction technique included the use of razor, roller mill, Moulinex blender and quartz sand with a mortar.

Most of these changes did not improve LOX recovery and had little effect on the good particulate recoveries obtained for LAH and AP. The only relationship observed was between LOX and plastids where both organelles gave low particulate recoveries. It has also been reported that triose phosphate isomerase activity was confined to the soluble fraction of soybean suspension

Table 2 Localization of enzymes in fractions obtained from potato shoots by differential centrifugation and the effect of high and low concentrations of $MgCl_2$

Enzyme localization	AP % activity		LAH % activity		NADH–cytochrome *c* reductase % activity	
	A	B	A	B	A	B
10 800 *g* 10 min pellet	8	5	8	6	9.5	6.5
38 000 *g* 20 min pellet	9	4	8	7.5	15.5	6.5
105 000 *g* 60 min pellet	12	15	7	14	9.5	10.5

Two potato shoot homogenates prepared with A, 10 mM $MgCl_2$ and 1 mM EDTA or B, 1 mM $MgCl_2$ and 10 mM EDTA in 8.5% w/w sucrose, 2.5% Ficoll, 4 mM cysteine, 1% BSA, 0.2 mM MBT and 0.1 M HEPES buffer, pH 7.4, were centrifuged at the stated *g* and the pellets obtained after each centrifugation were resuspended in the extraction buffer.

cultures [14] because the presence of starch in the proplastids of young cells made their isolation difficult.

Calabrese florets

LOX activity. Experiments using calabrese florets confirmed the previous work with cauliflowers that the LOX activity was localized in a high density body in floret material. The majority of activity in a particulate form was found after a 1500 *g* centrifugation. This sample also contained more chlorophyll than the other fractions. After a brief centrifugation on a linear sucrose density gradient (12000 *g* for 10 min) the density of the LOX band was 1.19 g cm^{-3}, intact chloroplasts 1.23 and broken chloroplasts 1.18g cm^{-3}.

Characterization of LAH activity

Previous work [15] had indicated that *p*-nitrophenyl palmitate was a suitable substrate for measurement of LAH activity and this substrate was used throughout this work. However, recent papers on potato tubers [16, 17] have indicated that more than one enzyme may be responsible for LAH activity. It was therefore necessary to check that the preparations used for the current work did in fact contain hydrolytic activity towards galactolipids and phospholipids as well as towards *p*-nitrophenyl palmitate. Incubation of monogalactosyl diglyceride or phosphatidylcholine with an homogenate from cauliflower florets or pea roots at pH 7.0 for 1 hr, resulted in a decrease in the acyl ester content of the substrates. The amount of enzyme added was limited by the endogenous lipid present and the need to add up to 2 μ equiv. of substrate per assay. Nevertheless, a small (5% of the added substrate) but consistent loss of acyl ester was recorded. Similar losses were recorded when a particulate fraction was incubated (1500–38000 *g* from florets and 38000 *g* pellet from roots) and when the active fractions from a sucrose or Ficoll gradient were combined and incubated with the two substrates.

The composition of lipid extracts obtained from freshly prepared homogenates of cauliflower florets and after incubation at 25° for 15 min showed changes, especially at a pH of 5.5. Both galactolipids and phospholipids disappeared during the incubation period and phosphatidic acid, which was present in the fresh homogenate, disappeared during the incubation.

DISCUSSION

The two enzyme activities studied here are involved in the breakdown of lipids in plant tissue but they are present in different subcellular fractions. From the three different tissues examined, the subcellular location of LAH activity is identical. It is not present in the plastids, mitochondria or microbodies but is in a low density body which in both sucrose and Ficoll gradients is close to the microsomal fraction. However, experiments with Mg^{2+} ions in the gradients show that the activity is not in the microsomal fraction. The coincident banding with AP, again in all three tissues, strongly suggests that it is present in a lysosomal particle or small vacuole. Cytological studies [18], suggest that lysosomal particles are equivalent to small vacuoles and histochemical studies [19–21] have shown acid hydrolases to be associated with vacuoles and tonoplasts.

The location of LOX is not as clearcut. In the previous paper we noted the wide range of subcellular fractions to which LOX activity had been ascribed by different workers with various plant materials. The present work has perhaps added to the confusion here. With floret material, LOX activity is localized in a heavy body similar to plastids and this similarity is seen from the work on potato shoots where it is impossible to achieve a high particulate recovery with LOX or triose phosphate isomerase. With pea roots however, the activity was coincident with LAH and AP and may therefore be present in 'lysosomes' or vacuoles. It should be emphasized that, due to the membrane-degrading properties of the enzymes under investigation, it was necessary in this work to adopt the unusual practice of using plant materials in which these activities were relatively low and thus would not cause destruction of the sensitive subcellular membrane structures during organelle isolation.

EXPERIMENTAL

Plant materials. Cauliflower, calabrese (*Brassica oleracea*), potato tubers (*Solanum tuberosum* cv Désirée) and pea seedlings (*Pisum sativum* cv Alaska) were grown and tissue collected as previously described [2]. Monogalactosyl diglyceride was prepared from spinach leaves [15].

Tissue extraction. Grinding material, for cauliflower florets, contained 0.3 M mannitol, 1 mM EDTA, 4 mM cysteine, 1% BSA and 0.1 M HEPES buffer. pH 7.4. With potato shoots and pea roots the mannitol was replaced by 8.5% w/w sucrose and 2.5% Ficoll and 2 mM 2-mercaptobenzothiazole was added to the medium. 1 mM $MgCl_2$ was added to the extraction and resuspension buffers when Mg^{2+} ions were used in gradients. Plant materials, after being shredded with a razor blade in ice-cold medium, were gently ground with fine sand with a pestle and mortar. Homogenate was passed through several layers of muslin and then centrifuged at the stated *g*. The crude particulate pellet was resuspended in the grinding buffer minus cysteine, MBT, BSA and Ficoll. Particulate preparations were used directly or layered on a linear sucrose or Ficoll gradient.

Density gradients. These were w/w sucrose solns, or of w/w Ficoll in 8.5% sucrose solns made up to 100% with 0.1 M HEPES buffer, pH 7.4 + 1 mM EDTA. Continuous gradients with cauliflower extracts were 5 ml of 58% sucrose, followed by 38 ml of a linear gradient from 58–16% with a final 5 ml of 16%. The gradient used for pea roots and potato shoots consisted of 5 ml 28.5% Ficoll, a linear gradient of 30 ml of 28.5–5%, followed by 10 ml 5% Ficoll. When appropriate 3 mM $MgCl_2$ was included. Other details were as previously described [2].

Enzyme assays. Catalase, triose phosphate isomerase, cytochrome oxidase, lipoxygenase and acid phosphatase as described earlier [2] NADH–cytochrome *c* reductase by the method of Donaldson *et al.* [6] at a pH of 7.0 and in the presence of antimycin A, isocitrate lyase, by the method of ref. [22] chlorophyll, by the method of ref. [23] and NADH–hydroxypyruvate reductase ref. [24]. Lipolytic acyl hydrolase activity was routinely determined using *p*-nitrophenyl-palmitate as substrate by incubation for 1 hr at 25° and subsequent determination of the *p*-nitrophenol released by reading against a second sample started at 60 min, after centrifuging both tubes if cloudy. Results expressed as nmol PNP hydrolysed. Due to the small amount of activity in the samples, the reaction could not be stopped by boiling or the addition of NaOH. The activity was also determined by loss of acyl ester. An aliquot of a $CHCl_3$ solution of monogalactosyl diglyceride or phosphatidylcholine containing about 1 μmol (2μ eq. acyl ester) of substrate was taken to dryness with 0.5 mg Triton X 100 in $CHCl_3$ and suspended in 0.6 ml HEPES buffer, pH 7.0. To this was added an aliquot of the enzyme prep. (0.5 g floret or root homogenate, 1.0 g of particulate or up to 2g particulate from a gradient) and water to 1.6 ml. The mixture was then incubated at 25°, with

shaking, for 60 min. To stop the reaction, 4 ml MeOH was added and the sample kept in a hot-water bath (80°) for 30 sec. Lipids in the enzyme digest were extracted [25] and 3 ml of the $CHCl_3$ layer taken to dryness and the acyl ester content determined by a slight modification [26] of the hydroxamate method [27].

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