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MICROSOMAL MEMBRANE LIPIDS* FROM PEA SHOOTS AND *RANUNCULUS SCELERATUS* PETIOLES

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Key Word Index—*Pisum sativum*; Leguminosae; pea; *Ranunculus sceleratus*; Ranunculaceae; membranes; lipids; composition.

Abstract—Microsomal membranes, relatively free of chloroplasts and mitochondria, were prepared from etiolated pea stems and *Ranunculus sceleratus* petioles by differential centrifugation. Relative quantitative analyses were made of the phospholipid, glycolipid, sterol and fatty acid contents.

INTRODUCTION

Hormone action in plants is being increasingly related to the properties of membranes. For example, Paleg and Wood [1, 2] have shown that the gibberellins can enhance the fluidity of lipid membranes and consequently permiability and membrane enzyme activity [3–5] and hypothesize that this may be the primary site of action in the plant. The primary effect of auxin in rapidly enhancing cell wall plasticity is seen as activating a membrane protein, a H^+ ion pump in the plasmalemma, which lowers the wall pH and consequently wall plasticity [6]. The speed of action of ethylene stimulated wall plasticity in water plants [7] and the ethylene inhibited plasticity in peas [8] was in the order of 10 min at room temperature, similar to the timing of the auxin response. It is again convenient to envisage a primary effect of the hormone in the membrane with a subsequent effect in the wall. For auxin and for ethylene a longer term effect on cell wall synthesis via the endoplasmic reticulum and golgi apparatus is also envisaged to back up the rapid effects on the wall plasticity.

In spite of the desire to invoke the membrane as the site of hormone action, very little is known of either the lipid or protein composition of specialised plant membranes, particularly those that are involved in cell wall modification and synthesis; the plasmalemma, the golgi and endoplasmic reticulum. This reflects the extreme difficulty in preparing these membranes as separate fractions for analysis. However, they can be easily isolated together as a microsomal fraction, free of chloroplasts and mitochondria. Thus we have prepared microsomal membranes from two plant sources and analysed them for phospholipid, glycolipid, sterol and fatty acid composition. Two systems were chosen that give opposite growth responses with ethylene, with the possibility in mind that major differences in composition might reflect aspects that are significant for hormone action. The two systems were etiolated pea shoots, where elongation is inhibited by ethylene [9, 10], and petioles of *Ranunculus sceleratus* where elongation is stimulated [11].

RESULTS

Membranes. Plate I shows electron micrographs of the organelles sedimenting at 20000 *g*. Homogenising etiolated pea shoots and *R. sceleratus* petioles for 3–5 sec in the polytron left most of the organelles intact (Figs. 1a and 1b) so that a short spin at 20000 *g* cleared the supernatant of mitochondria and chloroplasts leaving the microsomal fractions relatively free of contamination. As a measure of purification from chloroplasts and mitochondria we made use of chlorophyll and DPG as specific markers and calculated the absorption at 675 nm/PC, and DPG/PC ratios for the microsomal lipid extracts and total extracts. The absorption at 675 nm/PC ratio for the *R. sceleratus* microsome extract was 20 times less than for the total extract, and similarly, the DPG/PC ratio for the pea microsome extract was at least 22 times less than for the total extract. The mitochondrial outer membranes were not apparent in electron micrographs of the 20000 *g* pellet when it was fixed immediately after centrifugation but if before fixing, the pellet was equilibrated for 30 min in cold buffer containing 0.25 M sucrose, then they were apparent (Fig. 1a, inset). Therefore they were not contaminating the microsome fractions. The plasmalemmae in the microsomal fraction were highlighted for electron microscopy by specific staining with periodic, chromic and phosphotungstic acids [12] (Fig. 1e). We estimate that on average 30% of the vesicles were of plasmalemmae fragments. If EDTA was omitted from the homogenising medium, many of the vesicles seen in the 36000 *g* pellet were dotted with ribosomes, which we assume were fragments of the rough endoplasmic reticulum.

Lipids. The relative lipid compositions of the microsomal membranes are expressed in Table 1. Qualitatively, the microsomes are similar in that they contain the same classes of compounds though there is a notable absence

* Abbreviations used: SG = sterol glycosides, ESG = esterified sterolglycosides; Cer = cerebrosides; MGD and DGD = mono- and diglycosyldiglycerides; PC, PE, PI, PS, PG, PA and DPG = phosphatidylcholine, -ethanolamine, -inositol, -serine, -glycerol, phosphatidic acid and diphosphatidylglycerol respectively.

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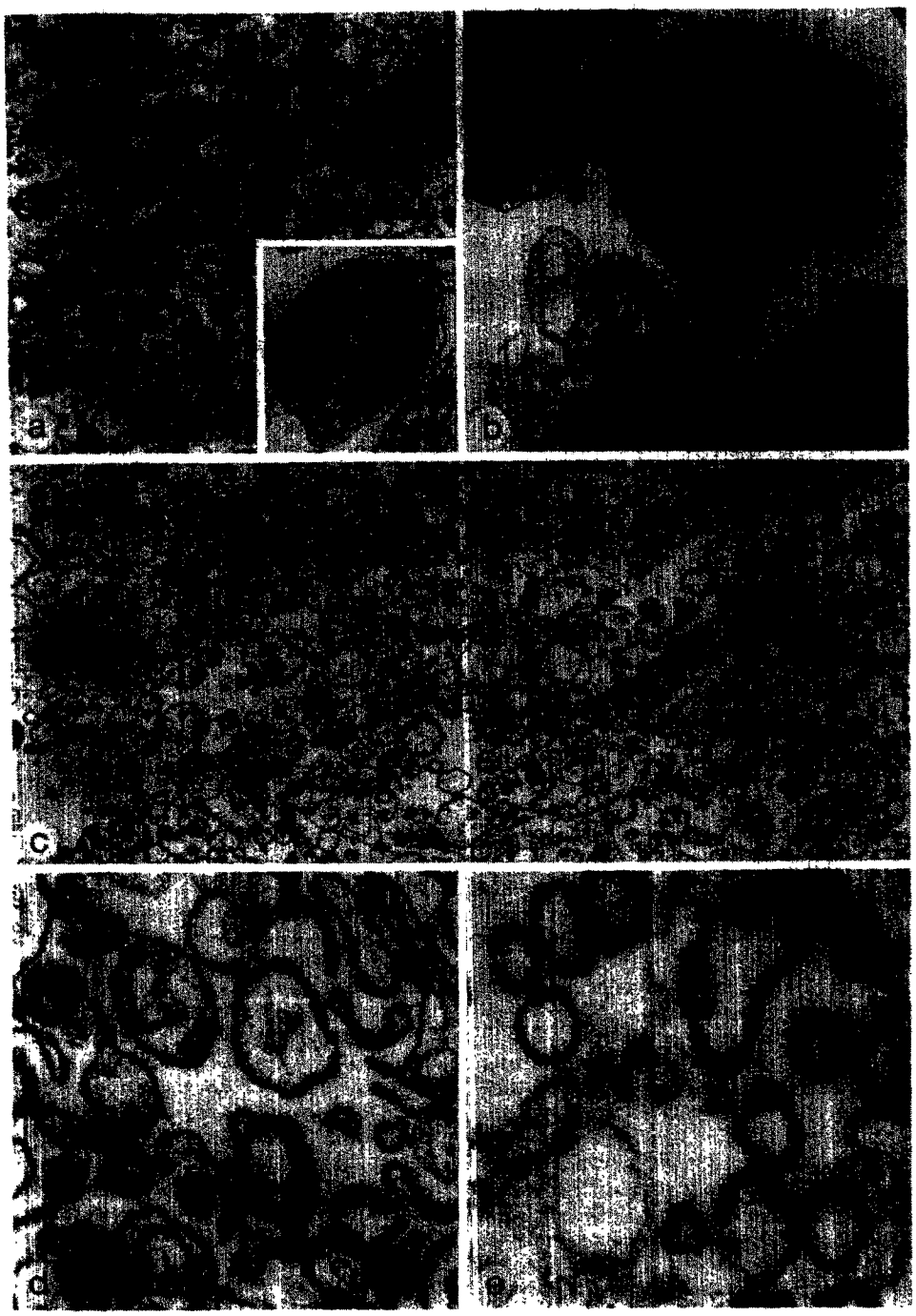

Fig. 1. Electron micrographs of the 20000 *g* pellets showing pea mitochondria (a, ×12960) and *R. sceleratus* chloroplasts (b, ×10960), and the 36500 *g* pellets showing *R. sceleratus* microsomal membranes (c, ×13600) and pea microsomal membranes (d and e, ×68240). The inset (a, ×34080) shows a pea mitochondrion after resuspending the 20000 pellet in 0.25 M glucose and buffer for 1 hr before fixing in gluteraldehyde. Plasmalemmae vesicles stained with PCAP (d) were more electron dense than other vesicles.

of DPG from both preparations, presumably because it was removed with the mitochondria for it was readily detected in total extracts. Quantitatively, the major difference between the two preparations lies in the relatively high level of MGD and DGD in *R. sceleratus* membranes compared with peas. Since chloroplasts are known to be very rich in these glycolipids the question arose again whether this was due to chloroplast contamination. In reassessing this possibility we analysed the DGD and MGD content of a total petiole extract. The MGD level was three times higher and the DGD level 1.6 times higher than in the microsomes (relative to PC). Thus the glycolipid content and MGD/DGD ratio of microsomes is lower than in whole tissue. The biggest

Table 1. Lipid composition of microsomal membranes from pea stems and *R. sceleratus* petioles

Lipid	% Lipid composition Pea	*R. sceleratus*
PC	28.9	19.8
PE	17 0	18.2
PI	6.1	3.3
PS	3.0	1 7
PG	4.2	6 0
PA	3 3	2.9
a	1 0	*t*
b	0.9	0 4
Total phospholipid	64.4	52.3
DGD	2.5	8.7
MGD	1.2	9.0
Cer	4 0	2 9
SG	3.0	2 3
ESG	2.8	1.5
Total glycolipid	13.5	24.4
Free sterol	22.1	23.4
Total sterol lipid	27.9	27.2

The relative molar compositions were calculated and expressed as percentages. *t* = trace.

difference between the phospholipids of the two membranes is in the PC level which is relatively low in *R. sceleratus* membranes since it alone complements most of the high level of glycolipid.

The relative sterol composition of the two microsomal fractions is similar when expressed quantitatively as free sterols, SG etc. but it is clear that pea microsomes contain a sterol, probably isofucosterol, which is absent from *R. sceleratus* microsomes (Table 2). In both preparations the sterol ester fraction contains a relatively high level of cholesterol and the free sterol fraction a relatively high level of stigmasterol, compared with the other fractions.

The fatty acid compositions of the two microsomal preparations differ in one major respect (Table 3): all the lipids of *R. sceleratus* microsomes contain a higher content of linolenic acid and a complementary lower level of linoleic acid. In addition, the fatty acid 20:0 is unique to pea membranes and 16:1 to *R. sceleratus* membranes.

DISCUSSION

The microsomal preparations were relatively free of chloroplast and mitochondrial contamination as witnessed by electron micrographs and chemical composition. Nonetheless, the preparations were a heterogeneous collection of membranes. Two major constituents were fragments of plasmalemmae and rough endoplasmic reticulum and we assume that tonoplast fragments were another major constituent with golgi and nuclear membranes as minor components. Most of the nuclear membranes were probably filtered off with the cell wall or pelleted at 20000 *g*.

The lipid compositions of the microsomes are not characterized by the presence of specific lipids; rather they are characterized by the lack of lipids typical of mitochondria and chloroplasts. Thus DPG is not detectable in microsome extracts and the MGD/DGD ratio is approximately 1, or less than 1, compared with values for chloroplast membranes as high as 2.4 [13], and cholesterol accounts for approximately 1% of the total sterol compared with 24% and 26% reported for chloroplasts and mitochondria respectively [14].

The lipid composition of pea microsomes differs markedly from that of *R. sceleratus* microsomes in three respects; a lower MGD + DGD/PC ratio, a lower linolenic acid content and the presence of what we think is isofucosterol. We were interested in whether these differences were related to the opposing effects of ethylene in these two systems. Applying the hypothesis that ethylene could affect the fluidity of a lipid membrane, as is claimed for the gibberellins [1, 2], then it is predicted that the lipid composition of *R. sceleratus* is such that in the presence of ethylene, fluidity is enhanced, as a consequence enzyme activity is enhanced [9, 11] and cell elongation stimulated. In contrast, the opposite is predicted for the effect of ethylene on the lipid membranes of peas. Thus what is being hypothesized for ethylene is similar to the known effect of cholesterol, which also has stimulating or restricting effects on the fluidity depending upon the other constituents of the membrane [15]. In order to test this hypothesis we have constructed liposomes from the lipids extracted from both pea and *R. sceleratus* microsomal membranes, after the manner of Wood and Paleg [1, 2], and looked for opposing effects of ethylene on the leakage rate of glucose and chromate from them. We have not found any effect, either positive or negative, on either of the liposomes. We therefore conclude that an effect of ethylene on lipid fluidity is not obvious and that the differences in composition between pea and buttercup are probably not related to the mode of action of ethylene.

Table 2. Sterol composition (as %) of free sterol, sterol glycoside and sterol ester fractions in pea and *R. sceleratus* microsomal membranes

Sterol	Pea free	Pea glycosides	Pea esters	*R. Sceleratus* free	*R. Sceleratus* glycosides	*R. Sceleratus* esters
Cholesterol	0.4	1 7	5 1	0.5	0.8	9.7
Campesterol	13.9	11 5	13 9	19.8	21.7	22 9
Stigmasterol	23.7	15.3	13.1	29.9	11.0	15 4
Sitosterol	46.1	56 9	39.6	51.5	55.7	44 5
Isofucosterol	10.1	3.9	15 4	0.0	0.0	0 0
others	5.8	10 7	12 9	2.3	10.7	7.5

The glycosidic fraction includes both SG and ESG.

Table 3. Fatty acid compositions (%) of pea and *R. sceleratus* microsomal membranes

Lipid		16:0	16:1	18:0	18 1	18:2	18:3	20:0
Pea	PC	22.9	0.0	3.4	2 2	66.5	5.0	0.0
	PE	35.4	0.0	3.8	2.8	54.4	3.4	0.0
	PI	64.5	0.0	4.0	4.3	25.3	1.9	0.0
	PG	63.4	0 0	2.1	2.1	29.5	2.9	0.0
	PS	23.9	0.0	6.5	1.3	45.7	5.5	17 1
	PA	42 8	0.0	4.5	2.4	45 6	2 1	2 6
	MGD + DGD	24.5	0 0	3.6	4.5	46 0	21.4	0 0
	Neutral lipid	28 8	0 0	4.5	7.9	52.7	6.1	0.0
R. sceleratus								
	PC	47.5	0 0	1.9	2.5	27.2	20.9	0.0
	PE	51.9	0 0	1.4	1.7	30 0	15.0	0.0
	PI	50.2	0.0	2.2	3.1	18.8	25.7	0.0
	PG	40.6	10.9	1.3	2.0	18.0	27.2	0.0
	PS	41.7	4.1	2.1	3 4	19.3	29 4	0 0
	PA	39 0	0.0	1.3	1.4	34.7	23.6	0 0
	MGD + DGD	19 9	0.0	*t*	2.4	11.3	57.0	0 0
	Neutral lipid	39.5	0.0	4.1	10.2	22 2	24.0	0.0

MGD + DGD % in *R. sceleratus* do not add up to 100%, 9.4% attributed to unknown compound with R_t 1.5 relative to methyl palmitate. *t* = trace.

EXPERIMENTAL

Plant material. *Pisum sativum* cv. Alaska seeds were germinated in sandwich boxes of sand in a growth room at 25° in the dark. When the shoots were approximately 10 cm above the sand they were cooled for 30 min at 4° before excising the apical buds and harvesting the stems for preparing membranes. *Ranunculus sceleratus* leaves were harvested from plants growing on the borders of ponds in the neighbourhood of Cambridge. They were also cooled for 30 min before excising the laminae and washing the petioles in ice-water prior to preparing membranes.

Microsome preparations. Each 100 g batch was initially chopped with a razor blade so that it was more accessible to the blades of a Polytron. It was then macerated in 300 ml buffer (0.35 M Sucrose, 0.001 M Na_2-EDTA, 0.05 M Tris, pH 7.6) for 3–5 sec in a Polytron high frequency homogenizer. The brei was filtered through 2 nappy liners and centrifuged at 2000/g or the supernatant was re-centrifuged at 36000 *g* were then pelleted in two alternative ways, either 50 ml 45% TCA was added, stirred for 5 min and the ppt. centrifuged at 2000/g or the supernatant was re-centrifuged at 36000 *g* for 20 min. All operations were performed below 5°.

Electron microscopy. Pellets were resuspended in 6% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.1 and centrifuged at 36000 *g*. Each pellet was washed 4 times over 2 hr in the buffer, fixed in 2% OsO_4, dehydrated through an ethanol series, then infiltrated with Spurr's Resin, unless they were to be stained with phosphotungstic acid–chromic acid, when they were infiltrated with TAAB resin. Sections were collected on uncoated grids and stained with uranium acetate followed by Reynold's lead citrate or stained with PTA-chromic acid [12] when the sections were collected on uncoated gold grids.

Lipid extraction. The membrane pellets were treated with boiling isopropanol and then stirred in isopropanol for 30 min at room temperature. Undissolved pellet was re-extracted for 30 min consecutively with $CHCl_3$–*iso*-PrOH (2:1) and $CHCl_3$–MeOH (2:1). The combined fractions were evaporated, taken up in $CHCl_3$, washed with 1% NaCl, dried, reevaporated, taken up in C_6H_6–EtOH (4:1) and stored frozen under N_2. Total lipid extracts were prepared in a similar manner, the intact plant tissue being cut into boiling *iso*-PrOH.

DEAE-cellulose column chromatography. Acidic lipids were separated from the rest on a short column of Whatman DE-52 after the method of Roughan and Batt [16] except that the acidic lipids were eluted with 27 ml $CHCl_3$–MeOH–H_2O (15:10:2) containing 200 mg NH_4COOH.

Thin layer chromatography. For qualitative and phospholipid analysis the 2-D solvent system (1) $CHCl_3$–MeOH–NH_3 (65:30:4) and (2) $CHCl_3$–MeOH–HoAc–H_2O (170:30:20:5) of Nichols and James [17] was used with Si gel H plus Na_2CO_3 [18]. Na_2CO_3 greatly improved the separation of PI and PS. For MGD and DGD analysis the above acid system was used with washed (1 N HCl, 1 N NaOH, MeOH, $CHCl_3$) Si gel H. The same washed support was used to separate cerebrosides with the solvent (3) $CHCl_3$–MeOH–H_2O (85:20:3). Free sterols were separated on Si gel G or Si gel $HF_{254+360}$ in (4) Et_2O–C_6H_6–EtOH–HoAc (40:50:2:0.2) [19]. Steryl glucosides were separated with washed Si gel H and (5) Et_2O–C_6H_6–EtOH–HoAc (30:50:8:0.2).

In general, spots or bands of lipids were visualized with I_2 or 25% H_2SO_4 at 120°. Free sterols on Si gel $HF_{254+360}$ were visualized as pale-blue bands under 360 nm light.

Gas liquid chromatography. Fatty acid methyl esters were separated on a 7′ column of 5% polyethylene glycol adipate on Chromasorb W HP, 120 mesh, at 190°, N_2 at 60 ml/min. Sterols were separated as acetates on SP-1000 [20]. Relative concentrations were calculated from the products of peak heights and retention times. The FID response was equivalent for all the fatty acids listed and was assumed to be equivalent for the sterol acetates. Lipids extracted for fatty acid analysis were kept under N_2 whenever possible e.g. when breaking vacuums and evaporating solvents from TLC media. The fatty acids in glycerolipids were methylated on the TLC media scraped from the plates using BF_3–methanol or H_2SO_4–methanol. Free sterols were eluted from Si gel with $CHCl_3$–MeOH (1:1) and acetylated with C_5H_5N–AcO. Glycosidic sterols were hydrolized on Si gel with 72% $HClO_4$ (0.23 ml in 25 ml THF) for 3 hr at 50°. The free sterol was then taken into $CHCl_3$, washed with 1% NaCl soln, the $CHCl_3$ evaporated and the sterol acetylated. Sterol esters were hydrolized with boiling 10% ethanolic KOH for 1 hr. The free sterols were recovered and acetylated as just described.

Quantitative analysis. Phospholipids were analyzed for phosphate [21], MGD, DGD and cerebrosides for galactose or glucose using phenol–H_2SO_4 [22] and free sterols for their ability to discolour chromic acid soln [23]. The phenol–H_2SO_4 reagent was also used for assaying steryl glycosides with synthetic cholesteryl glucoside [24] as standard.

Qualitative analysis. The lipids PC, PE, PI, PG, PA, PS, DPG, DGD and MGD were characterized by their behaviour on DEAE cellulose 2-D TLC, and their colour reaction with 25% H_2SO_4 at 120° and with the Dittmer–Lester phosphorous spray reagent [25] in comparison both with commercial standards and a plant lipid extract of known composition kindly supplied by Dr. B. W. Nichols. Two unidentified phospholipids were present in both membranes. The one labelled "a" chromatographed to a position equivalent to polyglycerophosphatide identified by Nicholls [26] in lettuce leaves. The other labelled "b" chromatographed proximal to MGD being relatively more mobile than MGD in the first of the 2-D systems and slightly less in the second. Galliard [27] similarly described a lipid in apples. What we assumed was sulpholipid was also present but like the phospholipid DPG in too low a concentration to quantitate. Cerebrosides were characterized by their similar chromatographic mobilities to commercial preparations and by their characteristic dull-purple colour on heating with H_2SO_4 at 120°. Free sterols and sterol esters were deemed to be those compounds that co-chromatographed with cholesterol and cholesteryl palmitate in TLC system 4. Sterol glycosides were characterized by co-chromatography with synthetic cholesterol glucoside in the above 2–D system. Esterified steryl glucosides were characterized as the only other group of compounds giving a positive Lieberman–Burchard reaction and having an R_f in system 4 equivalent to that reported by Clayton *et al.* [28]. The term neutral lipids in Table 3, is used to denote those that migrate with the solvent front in TLC system 2. Fatty acids were identified by the retention times of their methyl esters on PEGA compared with standards. Cholesterol, campesterol, stigmasterol and sitosterol were identified by their retention times on SP-100 compared with known sterol acetates. Isofucosterol acetate was tentatively identified by comparison with the data of Nordby and Nagy [19], who reported a retention time relative to cholesterol of 1.92. Under conditions in which the retention times for stigmasterol and sitosterol were practically identical to theirs, the retention time of the supposed isofucosterol was 1.90. There were several minor sterol components which we did not identify at all. Their retention times relative to sitosterol were 0.72, 1.06, 1.11 and 1.23. The last component was unique to peas, the others were common to both.

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