Pergamon

0031-9422(95)00111-5

Phytochemistry, Vol. 39, No. 6, pp. 1335–1345, 1995
Elsevier Science Ltd
Printed in Great Britain
0031-9422/95 $9.50 + 0.00

THE LIPID COMPOSITION OF CYTOSOLIC PARTICLES ISOLATED FROM SENESCING BEAN COTYLEDONS

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(*Received in revised form* 7 *December* 1994)

Key Word Index—*Phaseolus vulgaris*; Leguminosae; cotyledon; cytosol; sterols; steryl esters; wax esters.

Abstract—Non-sedimentable particles have been isolated from the cytosol of young two-day old and senescing seven-day-old bean cotyledons by ultrafiltration. The cytosolic particles have an average diameter of 240 nm and, when visualized in thin section by electron microscopy, appear osmiophilic and uniformly stained. Particles of similar size and morphology are also discernible in the cytoplasm of thin-sectioned cotyledon tissue. The cytosolic particles of both young and senescing tissue contain phospholipids with all of the fatty acids detectable in corresponding microsomal membrane phospholipids, and they also contain the same free sterols present in microsomal membranes. Free fatty acids and steryl/wax esters, phospholipid catabolites that are known to cause bilayer destabilization, are enriched in cytosolic particles relative to membranes by eight- to 23-fold and 41- to 213-fold for young and senescing cotyledon tissue, respectively. Thus phospholipid catabolite removal appears to be higher for the older membranes, but this notwithstanding, steryl/wax esters and free fatty acids still accumulate in microsomal membranes with advancing senescence. The data are consistent with the contention that blebbing of cytosolic particles from membranes is a means of removing phospholipid catabolites that would otherwise destabilize the bilayer, and suggest that the efficiency of catabolite removal is lower for senescing membranes.

INTRODUCTION

Membrane deterioration resulting in loss of function is an early and characteristic feature of tissue senescence [1]. In particular, previous studies with a number of senescing tissues including flower petals [2–4] and cotyledons [5, 6] have demonstrated that there is a selective depletion of phospholipid from senescing membranes. This engenders an enrichment of free sterols relative to phospholipid in membrane bilayers and a corresponding decrease in bulk lipid fluidity that has been measured by fluorescence polarization and electron spin resonance after labelling the membranes with lipid-soluble fluorescent and paramagnetic probes, respectively [6–8]. Phospholipid catabolism in senescing tissues appears to be mediated by the sequential actions of phospholipase D, phosphatidic acid phosphatase and lipolytic acyl hydrolase [9, 10]. The products of these reactions include phosphatidic acid, diacylglycerol and free fatty acids. As well, free linoleic and linolenic acids released by lipolytic acyl hydrolase serve as substrates for a membrane-associated lipoxygenase, which catalyses the formation of fatty acid hydroperoxides and initiates lipid peroxidation [7, 11]. The products of phospholipid catabolism are likely to engender destabilization of the bilayer if they accumulate in membranes. Phosphatidic acid and peroxidized lipids, for example, have been shown to act as Ca^{2+} ionophores, and diacylglycerol, peroxidized lipids and free fatty acids induce the formation of nonbilayer lipid configurations [12–14]. There are also reports that steryl esters and wax esters are formed from phospholipid catabolites by enzymes associated with microsomal membranes [15–19], and these too are nonbilayer lipids.

Senescing membranes also sustain lipid phase separations that give rise to coexisting domains of liquid-crystalline and gel phase lipid in the bilayer [20–22]. Indeed, packing imperfections at the phase boundaries render the bilayers leaky and are thought to account in part for the loss of membrane selective permeability that is a characteristic feature of senescence [22–24]. Earlier

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studies have indicated that these gel phase lipid domains contain phospholipid catabolites, in particular free fatty acids, that accumulate as senescence progresses [25]. Moreover, there is evidence to suggest that these catabolites are formed as well during normal membrane lipid turnover in nonsenescing tissue, but are removed from membrane bilayers in young tissue by a blebbing process that releases nonsedimentable cytosolic particles [25]. These cytosolic particles have been isolated from both plant and animal tissue, and they are enriched in membrane-destabilizing lipid catabolites and appear to serve as a vehicle for removing such catabolites from the bilayer [25–29]. They have been termed deteriosomes to connote this putative function [26]. Nonsedimentable cytosolic particles have been isolated from both young and senescing plant tissues [25, 26], although recent X-ray diffraction studies have indicated that the accumulation of lipid catabolites in senescing membranes and the consequent lateral phase separation of lipids may reflect partial impairment of particle blebbing with advancing senescence [25].

In the present study, we describe evidence indicating that nonsedimentable cytosolic particles isolated from senescing cotyledons of bean (*Phaseolus vulgaris*) contain phospholipid and free sterols, and are enriched in free fatty acids and steryl and wax esters. The lipids of the particles do not, however, appear to be organized in a bilayer.

RESULTS

Electron microscopy of stained whole mounts of isolated cytosolic particles indicated that they are spherical in nature, of variable size and have a propensity to fuse into larger particles (Fig. 1A). Light scattering measurements using a focused laser beam have indicated that the particles have an average diameter of 240 nm. In thin

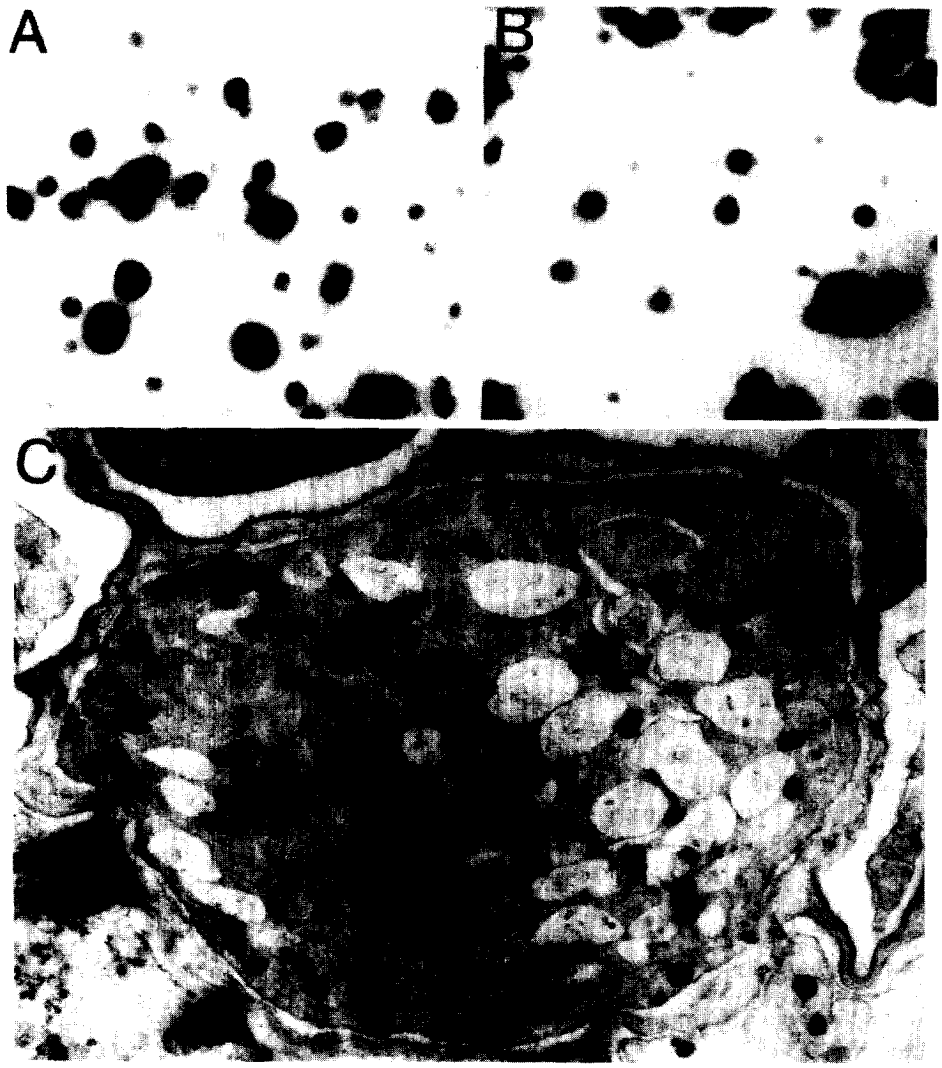

Fig. 1. Electron microscopy of cytosolic particles. (A), uranyl acetate-stained whole mount of particles isolated from nine-day-old cotyledon tissue; 46000 ×. (B), Thin section of particles isolated from two-day-old cotyledon tissue; 35000 ×. (C), Thin section of four-day-old cotyledon tissue showing *in situ* particles; 8968 ×.

section, the isolated particles are uniformly stained (Fig. 1B), and uniformly stained particles of similar size were also discernible in the cytoplasm of thin sectioned cotyledon tissue (Fig. 1C).

Lipid profiles obtained by thin layer chromatography for microsomal membranes and cytosolic particles isolated from young two-day-old and senescing seven-day-old cotyledon tissue are shown in Fig. 2. The microsomal membranes from two-day-old tissue feature a full spectrum of lipids including phospholipids, specifically phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid, and some neutral lipids. However, it is evident from the thin layer chromatogram that the majority of membrane lipid is phospholipid (Fig. 2A, lane 1). The cytosolic particles isolated from two-day-old cotyledons show a different pattern of lipid composition. Specifically, phospholipids are present, but neutral lipids in the high R_f region are predominant (Fig. 2A, lane 2). Microsomal membranes from seven-day-old cotyledons also have a full spectrum of lipids including phospholipids and a number of neutral lipids (Fig. 2B, lane 1). Cytosolic particles from seven-day-old cotyledons have a lipid composition that is similar to that of cytosolic particles from two-day-old tissue in that some phospholipid is present, but there are much higher concentrations of neutral lipids (Fig. 2B, lane 2).

The phospholipids in the isolated cytosolic particles contain all of the fatty acids present in corresponding microsomal membranes including palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) (Table 1). Moreover, for young two-day-old tissue, the fatty acid composition of phospholipids from microsomal membranes closely resembles that for phospholipids from the cytosolic particles. Palmitic acid (16:0) is the dominant phospholipid fatty acid in each case comprising 44% of the total for membranes and 36% of the total for the cytosolic particles, and the unsaturated fatty acids, 18:1, 18:2 and 18:3, collectively comprise 51% and 55% of the total for microsomal membrane phospholipid and cytosolic particle phospholipid, respectively (Table 1). Indeed, the phospholipid saturated:unsaturated fatty acid ratio proved to be 0.98 for microsomal membranes from two-day-old tissue and 0.82 for corresponding cytosolic particles (Fig. 3A). With advancing senescence, however, differences in the fatty

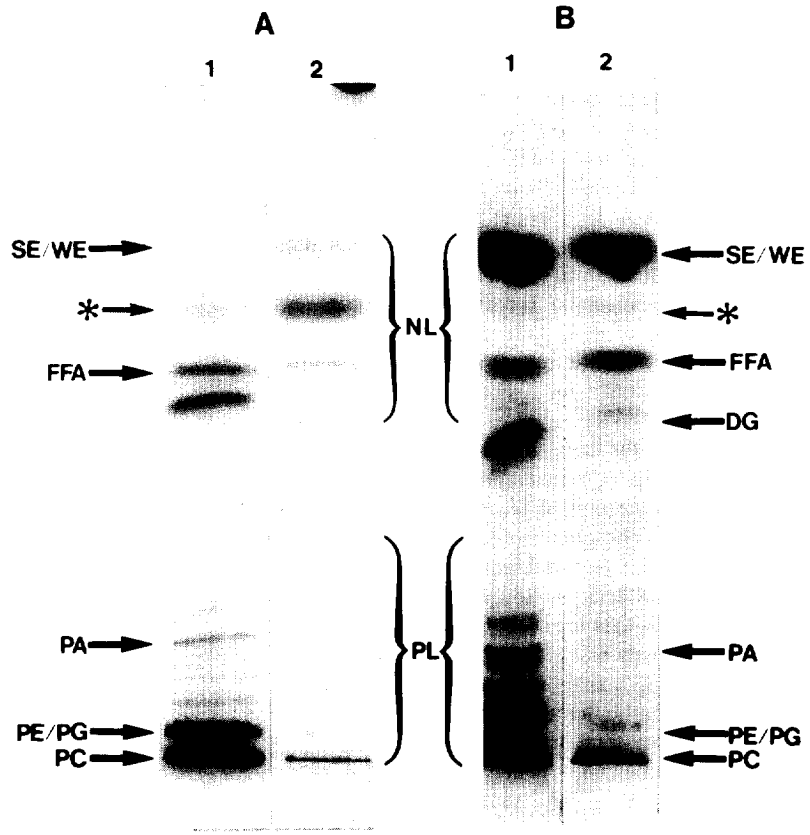

Fig. 2. Thin layer chromatography of lipids extracted from microsomal membranes and cytosolic particles. (A) Isolated from two-day-old cotyledons: lane 1, lipids from microsomal membranes (0.5 mg protein equivalent); lane 2, lipids from cytosolic particles (1 mg protein equivalent). (B) isolated from seven-day-old cotyledons; lane 1, lipids from microsomal membranes (2.0 mg protein equivalent); lane 2, lipids from cytosolic particles (4.0 mg protein equivalent). WE/SE, Wax esters and steryl esters; FFA, free fatty acid; PA, phosphatidic acid; DG, diacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; *unknown; NL, neutral lipids. Data are representative of five separate experiments showing the same results.

Table 1. Fatty acid composition of phospholipids isolated from microsomal membranes and cytosolic particles of young and senescing bean cotyledons

Source of lipid	Mole%				
	16:0	18:0	18:1	18:2	18:3
Microsomal membranes from two-day-old cotyledons	44.2 ± 2.6	5.2 ± 0.5	16.1 ± 0.7	22.9 ± 1.7	11.8 ± 1.0
Microsomal membranes from seven-day-old cotyledons	45.2 ± 2.2	6.1 ± 0.2	11.7 ± 0.4	32.5 ± 0.5	8.9 ± 0.2
Cytosolic particles from two-day-old cotyledons	36.1 ± 5.1	8.9 ± 3.8	19.0 ± 1.0	22.3 ± 1.4	13.7 ± 3.1
Cytosolic particles from seven-day-old cotyledons	38.3 ± 0.2	23.4 ± 1.0	14.4 ± 1.0	10.2 ± 1.1	13.3 ± 1.1

Standard errors of the means are indicated for $n = 3$. 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

acid composition of microsomal phospholipid and cytosolic particle phospholipid became apparent. Specifically, although in senescing seven-day-old tissue 16:0 remains the dominant fatty acid in both cases, accounting for 45% and 38% of the total for microsomes and cytosolic particles, respectively, the unsaturated fatty acids, 18:1, 18:2 and 18:3 comprise only 37% of the total for particle phospholipid compared to 54% of the total for microsomal phospholipid (Table 1). This reflects an increase in 18:0 and a decrease in 18:2 in the phospholipid of the cytosolic particles as senescence progresses (Table 1). The saturated:unsaturated fatty acid ratio remained essentially unchanged for microsomal membrane phospholipid with advancing senescence, but for cytosolic particles rose from 0.82 for young two-day-old tissue to 1.61 for senescing seven-day-old tissue (Fig. 3A).

Cytosolic particles also contain the same free sterols that are present in corresponding microsomal membranes, specifically campesterol, stigmasterol, sitosterol, isofucosterol and small amounts of cholesterol (Table 2). As well, for both young two-day-old tissue and senescing seven-day-old tissue the free sterol composition of the cytosolic particles is comparable to that for corresponding microsomal membranes. In each case, stigmasterol and sitosterol are the dominant sterols comprising 60–80% of the total (Table 2). Of particular interest is the finding that the proportion of stigmasterol in microsomal membranes rises with advancing senescence from 35% in young two-day-old tissue to 52% in senescing seven-day-old tissue, and that an increase in stigmasterol with advancing senescence of similar magnitude is also evident in the cytosolic particles (Table 2). As well, the sterol:phospholipid fatty acid ratio is higher in the cytosolic particles than in corresponding microsomal membranes by ~1.4-fold and ~1.7-fold for young two-day-old and senescing seven-day-old tissue, respectively (Fig. 3B).

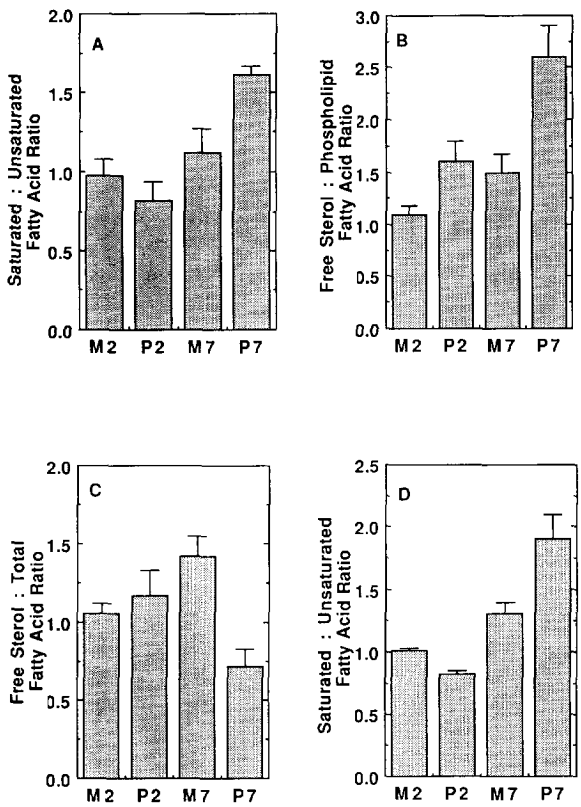

Fig. 3. Lipid characteristics of microsomal membranes and cytosolic particles from young two-day-old and senescing seven-day-old bean cotyledons. (A) Phospholipid saturated:unsaturated fatty acid ratios; (B) free sterol:phospholipid fatty acid ratios; (C) free sterol:total fatty acid ratios; (D) saturated:unsaturated total fatty acid ratios. (M2) Microsomal membranes from two-day-old cotyledons; (P2) cytosolic particles from two-day-old cotyledons; (M7) microsomal membranes from seven-day-old cotyledons; (P7) cytosolic particles from seven-day-old cotyledons. Standard errors of the means are indicated for $n = 3$.

Table 2. Free sterol composition of microsomal membranes and cytosolic particles from young and senescing bean cotyledons

	Mole%			
	Membrane		Cytosolic particles	
Sterol	two-day	seven-day	two-day	seven-day
Cholesterol	1.6 ± 0.6	1.0 ± 0.2	5.9 ± 2.3	7.6 ± 2.0
Campesterol	5.7 ± 1.1	5.4 ± 1.3	8.3 ± 3.1	12.7 ± 2.9
Stigmasterol	34.8 ± 0.8	51.8 ± 5.3	22.6 ± 3.0	42.1 ± 2.8
Sitosterol	41.6 ± 1.4	32.3 ± 6.9	42.3 ± 2.6	21.4 ± 5.3
Isofucosterol	15.1 ± 0.1	8.7 ± 0.4	11.3 ± 2.6	16.0 ± 3.4
Unknown	0.3 ± 0.1	1.5 ± 1.2	ND	8.3 ± 0.5

Standard errors of the means are indicated for $n = 3$.
ND, Not detectable.

Phospholipids and sterols are arranged amphipathically in membrane bilayers such that the sterols associate with phospholipid fatty acids and modulate lipid fluidity [6, 7, 20]. The sterol: total fatty acid ratio for microsomal membranes increases by ~34% between days 2 and 7 (Fig. 3C), and this correlates with an ~20% increase in DPH polarization reflecting a decrease in membrane lipid fluidity (Fig. 4). Lipid fluidity is also affected by fatty acid saturation [7], and in this context the ~30% increase in the saturated:unsaturated fatty acid ratio for the total microsomal fatty acid complement between days 2 and 7 (Fig. 3D) presumably also contributes to the decrease in membrane lipid fluidity detectable by fluorescence depolarization during this period (Fig. 4). For cytosolic particles, however, the DPH polarization values are only slightly lower than those for corresponding microsomal membranes for both young two-day-old and senescing seven-day-old tissue, notwithstanding large differences, especially for the older tissue, in fatty acid saturation and relative levels of free sterols. For seven-day-old tissue, for example, the DPH polarization value reflecting bulk lipid fluidity is only 7% lower in the cytosolic particles than in corresponding microsomal membranes (Fig. 4). Yet the cytosolic particle fatty acid saturation level is ~45% higher than the corresponding value for microsomal membranes (Fig. 3D), and the sterol:fatty acid ratio for the cytosolic particles is ~49% lower than the corresponding value for microsomal membranes (Fig. 3C). This can be interpreted as indicating that the free sterols and levels of fatty acid saturation are exerting the same influence on bulk lipid fluidity in the cytosolic particles as they do in membranes. That is, the propensity for the increase in saturation to decrease lipid fluidity appears to be largely offset by the decrease in sterol:fatty acid ratio, which would normally increase bulk lipid fluidity.

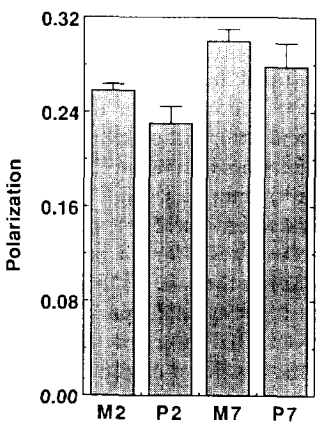

Fig. 4. Bulk lipid fluidity of microsomal membranes and cytosolic particles from young two-day-old and senescing seven-day-old bean cotyledons. Fluidity was determined by measuring DPH polarization. (M2) Microsomal membranes from two-day-old cotyledons; (P2), cytosolic particles from two-day-old cotyledons; (M7), microsomal membranes from seven-day-old cotyledons; (P7), cytosolic particles from seven-day-old cotyledons. Standard errors of the means are indicated for $n = 3$.

This contention is reinforced by a comparison of the change in cytosolic particle DPH polarization during senescence with corresponding changes in cytosolic particle fatty acid saturation and relative sterol concentration. For example, DPH polarization for the cytosolic particles increases by ~22% between days 2 and 7 as the cotyledons senesce (Fig. 4) reflecting a decrease in bulk lipid fluidity. Yet, the sterol:total fatty acid ratio declines by ~38% during the same period (Fig. 3C), which would normally result in an increase in bulk lipid fluidity. However, the increase in DPH polarization does correlate with an ~131% increase in fatty acid saturation (Fig. 3D) that appears to offset the counter influence of the declining free sterol complement. These observations collectively suggest that the free sterols in the cytosolic particles are able to interact with fatty acids and influence their rotational motion in much the same manner as in membrane bilayers.

The neutral lipids present in cytosolic particles and in microsomal membranes were identified by co-chromatography with authentic standards. Those that could be definitively identified in this manner included free fatty acids and diacylglycerol (Fig. 2). In addition,

there was a neutral lipid band in the thin layer chromatograms that could not be identified by cochromatography with standards (Fig. 2). The highest R_f neutral lipid band in the thin layer chromatograms was found to comigrate with steryl ester and wax ester standards in the solvent system used. As well, free sterols and free long-chain alcohols were formed during the reaction of this fraction with boron trifluoride/methanol and were identified by cochromatography with authentic standards (Fig. 5). The fatty acid methyl esters corresponded to fatty acids found in corresponding microsomal membranes, and included palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid.

Levels of ester fatty acid, free fatty acid and phospholipid fatty acid in smooth microsomal membranes and cytosolic particles isolated from young two-day-old and senescing seven-day-old cotyledons were quantified on a per cotyledon basis and also relative to protein (Table 3). For both ages of tissue, ester fatty acids, free fatty acids and phospholipid fatty acids were lower in cytosolic particles than in corresponding microsomal membranes (Table 3). This can be attributed to the lower abundance of cytosolic particles than of membranes. These data were in turn used to calculate the amounts of free and ester fatty acid relative to phospholipid fatty acid in microsomal membranes and cytosolic particles (Table 4). From these ratios, the relative enrichment of the free fatty acid pool and ester fatty acid pool with respect to phospholipid fatty acid were calculated. In the case of free fatty acids, there is an 8.8-fold enrichment for two-day-old tissue and a 23.8-fold enrichment for seven-day-old tissue in cytosolic particles relative to smooth microsomes (Table 4). For ester fatty acids, the corresponding enrichments are 41.5-fold for two-day-old tissue and 213-fold for seven-day-old tissue (Table 4).

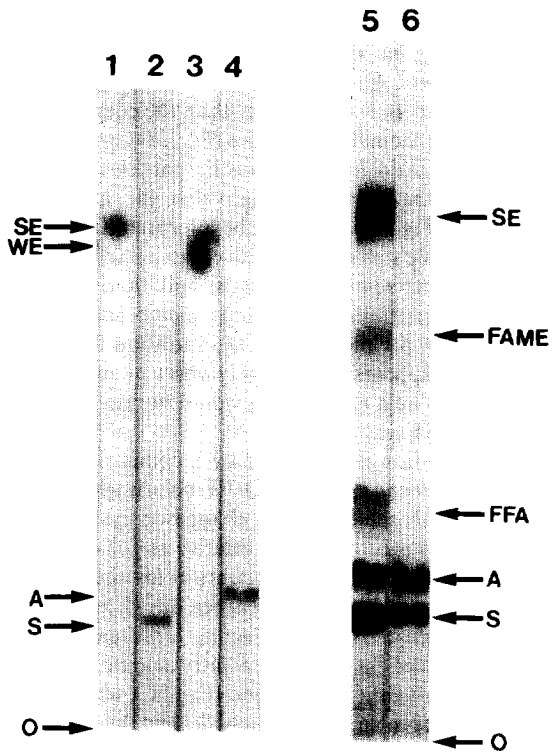

Fig. 5. Thin layer chromatograms showing boron trifluoride hydrolysis products of steryl ester (cholesterol palmitate), wax ester (palmitoyl oleate) and the highest R_f neutral lipid fraction purified from cytosolic particles (30 mg protein equivalent) isolated from two-day-old cotyledons. Lane 1, steryl ester; lane 2, steryl ester after reaction with boron trifluoride/methanol; lane 3, wax ester; lane 4, wax ester after reaction with boron trifluoride/methanol; lane 5, standards; lane 6, neutral lipid fraction after boron trifluoride/methanol treatment. O, Origin; S, sterol (sitosterol); A, long chain alcohol (oleoyl alcohol); WE, wax ester (palmitoyl oleate); SE, steryl ester (cholesteryl palmitate); FAME, fatty acid methyl ester (oleic acid methyl ester); FFA, free fatty acid (oleic acid).

Compositional studies of the fatty acids in the ester, free fatty acid and phospholipid fatty acid pools were also carried out for cytosolic particles and microsomes isolated from two- and seven-day-old tissue. It is clear from the data in Fig. 6 that the fatty acid compositions of these fractions are distinguishable in both cytosolic particles and smooth microsomes from two-day-old cotyledons. For cytosolic particles, for example, the ester fatty acid pool shows a predominance of saturated fatty acids, with palmitic acid and stearic acid combined comprising 74% of the total fatty acids in this pool (Fig. 6B). By contrast, these two saturated fatty acids account for only 39 and 34% of the total fatty acid complement in the phospholipid and free fatty acid pools, respectively (Fig. 6B). In addition, there are differences in composition for the respective pools between cytosolic particles and microsomes (Fig. 6). For the ester fraction, for example, stearic acid and palmitic acid together accounted for 55% of the total for microsomal membranes compared with 74% for cytosolic particles (Fig. 6). Palmitoleic acid (16:1), which is present in plastid membranes, was only detectable in the free and ester fatty acid pools (Fig. 6A). Similar trends were evident for cytosolic particles and smooth microsomal fractions isolated from senescing seven-day-old cotyledons. Again, there were clear differences in fatty acid composition among the phospholipid, free and ester fatty acid pools for cytosolic particles and for microsomal membranes (Fig. 7). For cytosolic particles, the saturated fatty acids, stearic and palmitic, were the dominant fatty acids for all of the pools (Fig. 7B), whereas this was not the case for the smooth microsomes (Fig. 7A). As well, for cytosolic particles and microsomal membranes from the seven-day-old cotyledons, palmitoleic acid (16:1) was not detectable in any of the fatty acid pools (Fig. 7).

The composition of the ester fatty acid pool in cytosolic particles changed with advancing senescence (Fig. 8A). In particular, linoleic acid (18:2) was not detectable in the ester fraction from cytosolic particles of young two-day-old cotyledons, whereas linolenic acid (18:3) was not detectable in the ester fraction of cytosolic particles isolated from senescing seven-day-old cotyledons (Fig. 8A). There were also changes in the composition of the free fatty acid pool of cytosolic particles with

Table 3. Levels of ester fatty acid, free fatty acid and phospholipid fatty acid in microsomal membranes and cytosolic particles from cotyledon tissue

	Two-day-old tissue				Seven-day-old-tissue			
	μg FA/cotyledon		μg FA/mg protein		μg FA/cotyledon		μg FA/mg protein	
Lipid fraction	Microsomes	Cytosolic particles	Microsomes	Cytosolic particles	Microsomes	Cytosolic particles	Microsomes	Cytosolic particles
Ester FA	0.15 ± 0.04	0.04 ± 0.002	0.75 ± 0.01	0.44 ± 0.07	0.05 ± 0.005	0.02 ± 0.002	1.81 ± 0.28	1.14 ± 0.01
Free FA	5.03 ± 1.32	0.20 ± 0.02	13.13 ± 0.43	1.53 ± 0.07	0.46 ± 0.09	0.03 ± 0.003	17.94 ± 0.89	1.17 ± 0.45
Phospholipid FA	113.27 ± 1.84	0.77 ± 0.08	392.98 ± 33.06	5.27 ± 0.87	7.03 ± 0.08	0.03 ± 0.001	324.64 ± 24.20	0.89 ± 0.001

Means ± s.e. are shown for $n = 3$.
Ester fatty acid includes steryl esters and wax esters.
FA, fatty acid.

advancing senescence (Fig. 8B). In particular, there was a greater proportion (46% as compared with 11%) of polyunsaturated free fatty acids (linoleic and linolenic) in cytosolic particles from two-day-old cotyledons than in those from seven-day-old cotyledons (Fig. 8B). Finally, the fatty acid composition of cytosolic particle phospholipids also changed as the cotyledons aged (Fig. 8C). More specifically, the phospholipids of the older seven-day-old cytosolic particles contained a higher proportion of saturated fatty acids than those of the younger two-day-old cytosolic particles (Fig. 8C).

DISCUSSION

It is apparent from the present study that there are three distinguishable fatty acid pools in microsomal membranes from both young and senescing tissue, phospholipid fatty acids, free fatty acids and steryl/wax ester fatty acids, respectively. The steryl/wax ester fatty acid complement in membranes is small amounting to $< 1\%$ of the phospholipid fatty acid complement for young and senescing membranes, although it is noteworthy that levels of steryl/wax ester fatty acids relative to membrane protein increase by ~ 2.5-fold with advancing senescence. Steryl and wax esters appear to be formed from phospholipid catabolites. There is evidence, for example, that 1,2-diacylglycerol serves as an acyl donor for steryl ester synthesis in spinach leaves [15]. Earlier studies have indicated that the enzymes mediating the formation of both steryl and wax esters are membrane-associated. Avato [16] found three-fold more wax ester synthesizing activity associated with microsomal membranes from barley leaves than in a corresponding soluble fraction. Kalinowska *et al.* [17, 18] reported steryl ester synthesizing activity in a 300–16 000 *g* pellet from homogenates of white mustard seedling roots, and Garcia and Mudd [19] have reported on the basis of detailed fractionation studies that 16% of the cellular steryl ester biosynthesis activity in spinach leaves is localized in chloroplasts, 21% in mitochondria, 57% in a microsomal fraction and only 5% in the cytosolic fraction. It has also been reported that there is reversible enzymatic transesterification between wax esters and steryl esters [17].

These distinguishable fatty acid pools are also present in nonsedimentable particles isolated from the cytosol of young and senescing cotyledon tissue. Electron microscopy of thin-sectioned cytosolic particles and of thin-sectioned cotyledon tissue has indicated that the particles are uniformly osmiophilic, and there is no evidence for a lipid bilayer. This suggests that the two fracture planes noted earlier in freeze-fracture electron micrographs of the particles [26] may reflect an external monolayer of phospholipid as has been proposed for oil bodies [30]. Indeed, apart from being much smaller, the cytosolic particles bear a morphological resemblance to plastoglobuli present in the chloroplast stroma of senescing leaves [31–35]. Plastoglobuli are thought to be formed during the dissolution of thylakoids accompanying senescence and range from 80 to 1250 nm in diameter [34],

Table 4. Enrichment of free fatty acids and ester fatty acids in cytosolic particles relative to microsomal membranes

	Ester FA/phospholipid FA			Free FA/phospholipid FA		
Tissue age	Micros	Particle	Enrich	Micros	Particle	Enrich
Two-day	0.002	0.083	41.5	0.033	0.29	8.8
Seven-day	0.006	1.28	213	0.055	1.31	23.8

Ratios were derived from the data (μg fatty acid/mg protein) in Table 3.
Micros, microsomal membranes; particle, cytosolic particles; enrich, enrichment (cytosolic particle/microsomal membrane).

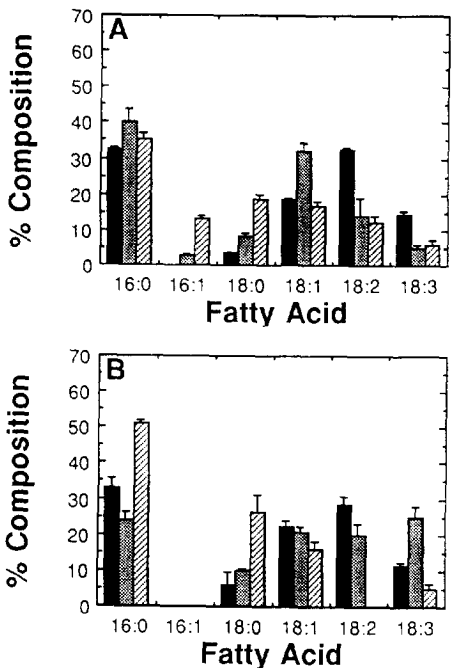

Fig. 6. Fatty acid composition (W/W) of the phospholipid, free and ester fatty acid fractions isolated from microsomal membranes (A) and cytosolic particles (B) of two-day-old cotyledons. Means ±s.e. for $n = 3$ separate experiments are shown. (Solid bars), phospholipid fatty acids; (stippled bars), free fatty acids; (hatched bars), ester fatty acids. 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0 stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

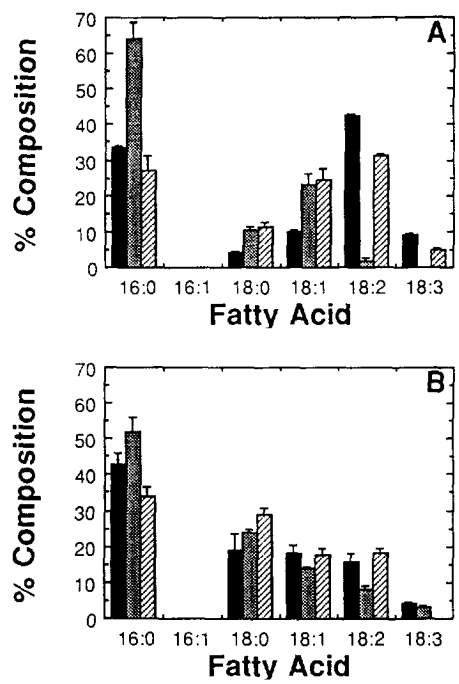

Fig. 7. Fatty acid composition (W/W) of the phospholipid, free and ester fatty acid fractions isolated from microsomal membranes (A) and cytosolic particles (B) of seven-day-old cotyledons. Means ± s.e. for $n = 3$ separate experiments are shown. (Solid bars), phospholipid fatty acids; (stippled bars), free fatty acids; (hatched bars), ester fatty acids. 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0 stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

whereas the cytosolic particles have an average diameter of only 240 nm.

The finding that steryl/wax ester fatty acids and free fatty acids identical to those found in membrane phospholipids (i.e. palmitic, stearic, oleic, linoleic and linolenic acids) are present in larger amounts, relative to phospholipid, in cytosolic particles than in corresponding microsomal membranes suggests that these phospholipid catabolites are removed from membranes by blebbing of cytosolic particles. Indeed, the blebbing process may be a means of selectively removing phospholipid catabolites that would otherwise destabilize the bilayer. Free fatty acids, for example, are known to induce a lateral phase separation of membrane lipids [25], and steryl esters are also thought to cause bilayer disorganization [36]. The notion that these cytosolic particles originate from membranes is reinforced by the finding that the cytosolic particles and the membranes both contain three distinct fatty acid pools, phospholipid fatty acids, free fatty acids and steryl/wax ester fatty acids, respectively, and the same five fatty acids, palmitic, stearic, oleic, linoleic and

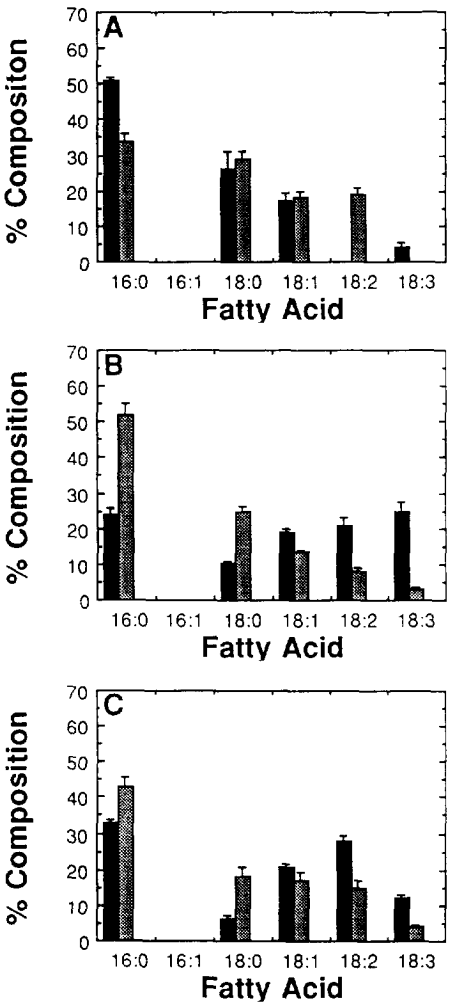

Fig. 8. Senescence-related changes in the fatty acid composition (W/W) of the ester fatty acid pool (A), free fatty acid pool (B) and phospholipid fatty acid pool (C) of cytosolic particles. Means ± s.e. are shown for $n = 3$. (Solid bars), cytosolic particles from young two-day-old cotyledon tissue; (stippled bars), cytosolic particles from senescing seven-day-old cotyledon tissue. 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0 stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

linolenic acids, are present in all three pools in membranes and cytosolic particles, although in varying proportions. As well, the cytosolic particles contain free sterols, indeed the same free sterols that are found in corresponding microsomal membranes. Moreover, judging from measurements of DPH polarization the free sterols in cytosolic particles are able to influence bulk lipid fluidity, presumably by associating with fatty acids, in much the same manner as in membrane bilayers. It is apparent, however, that selective components of the membrane bilayers are removed by the blebbing process. In particular, the cytosolic particles are enriched in free sterols, free fatty acids and steryl/wax esters by comparison with corresponding microsomal membranes. As well, the phospholipids of cytosolic particles are more saturated than their membrane counterparts, particularly in senescing seven-day-old tissue. These observations support the contention that the blebbing process is selective, for if it were random the lipid composition of the particles should not be different from that of corresponding membranes.

The enrichment of phospholipid catabolites in cytosolic particles relative to membranes increases with age. For young two-day-old cotyledon tissue, the cytosolic particles are enriched by 41.5-fold in ester fatty acids and 8.8-fold in free fatty acids relative to phospholipid by comparison with microsomal membranes, whereas for senescing seven-day-old tissue, the corresponding enrichments are 213-fold for ester fatty acids and 23.8-fold for free fatty acids. Thus phospholipid catabolite removal from membranes appears to be higher for senescing seven-day-old tissue than for young two-day-old tissue in that there is a very much higher proportion of steryl/wax esters and free fatty acids relative to phospholipid in cytosolic particles from the older tissue. This notwithstanding, steryl/wax esters and free fatty acids accumulate in microsomal membranes as senescence progresses. Indeed, the concentration of steryl/wax ester fatty acid and free fatty acid in microsomal membranes, relative to phospholipid, increased by three-fold and 1.7-fold, respectively, between days 2 and 7 as the cotyledons aged. This suggests that, notwithstanding the higher concentration of phospholipid catabolites relative to phospholipid in cytosolic particles from the older tissue, the blebbing process is unable to remove all of the catabolites from the older membranes. Indeed, previous studies have indicated that the accumulation of these phospholipid catabolites in older membranes engenders a lateral phase separation of bilayer lipids that contributes to membrane dysfunction [21, 22, 25].

EXPERIMENTAL

Fractionation procedures. Bean seeds of *Phaseolus vulgaris* L. (cv Kinghorn) were germinated in vermiculite at 27° and 90% relative humidity under conditions of etiolation. Cotyledons were harvested at 2, 4, 7 or 9 days after planting. The cotyledons were homogenized in 50 mM $NaHCO_3$–0.3 M sucrose, pH 7.0, and smooth microsomal membranes were isolated by gradient centrifugation [6]. Nonsedimentable cytosolic particles were isolated from the post-microsomal supernatant by ultrafiltration through a 300000 dalton cut-off filter (Omegacell, Pharmacia) [26].

Analytical procedures. Lipids were extracted from cytosolic particles or microsomal membranes as described [37]. The extracted lipids were spotted on to silica gel 60 TLC plates (Merck) and developed in $CHCl_3$–HOAc–MeOH–H_2O (70:25:5:2) for the first 5 cm, dried under N_2 and then completely developed in petrol–Et_2O–HOAc (70:30:1). The separated lipids were visualized with I_2 vapour or by H_2SO_4 charring and identified by co-chromatography with authentic standards.

Separated phospholipids, free fatty acids and wax and steryl esters were extracted from the silica gel with two washings of 2.5 ml of $CHCl_3$–MeOH–H_2O (2:1:0.8). An additional 1.5 ml $CHCl_3$ and 2 ml H_2O were added, and the $CHCl_3$ layer was removed and dried under N_2. Free fatty acids were methylated directly as described by Morrison and Smith [38]. Phospholipid and steryl and wax ester fatty acids were transmethylated in boron trifluoride [38]. Fatty acid methyl esters were quantified by co-chromatography with an internal standard using a Hewlett–Packard model 5890 gas chromatograph equipped with a flame ionization detector and a 15 m × 0.25 i.d. fused silica capillary column (DB-23 J&W Scientific) operated isothermally at 175°. Integration was performed using a Hewlett-packard model 3396A integrator. The separated peaks were identified by co-chromatography with authentic standards.

In some experiments, the lipid extracts were fractionated into neutral lipids and phospholipids on silica Sep-Pak columns as described by Hamilton and Comai [39]. The neutral lipid fraction containing free sterols was eluted from Sep-Pak column in $CHCl_3$ [39]. The free sterols were further purified by TLC on silica gel 60 plates (Mandel) in petrol–Et_2O–HOAc (70:30:1), identified using authentic cholesterol (Sigma) as a standard, eluted by washing the scraped silica gel twice with $CHCl_3$–MeOH (4:1) and once with Et_2O, and derivatized according to Grunwald [40]. Desmosterol was added as an internal standard before derivatization. The derivatized sterols were analysed on a GLC (Hewlett Packard, Model 5890) equipped with a flame ionization detector and a capillary column (OV-17, 15 m × 0.25 mm i.d.) (J and W Scientific). The GLC was operated isothermally with the column, injector and detector temperatures at 275°, 300° and 350°, respectively.

Fluidity measurements. Bulk lipid fluidity of microsomal membranes and cytosolic particles was determined by measuring fluorescence polarization after labelling the membranes and the particles with diphenyl hexatriene (DPH) as described previously [7]. DPH polarization measurements were made at 23° with an SLM spectrofluorimeter (model 8000).

Electron microscopy and light scattering measurements. The isolated cytosolic particles were sized by dynamic light scattering measurements using a helium/neon laser (model 125; Spectra-Physics) that was focused into a thermally jacketed scattering chamber [41]. For electron microscopy, droplets of cytosolic particle suspension were placed on copper grids that had been coated with Formvar, and the sample was dried with the edge of a piece of filter paper and stained for 5 sec with 2% uranyl acetate. The isolated cytosolic particles were also thin-sectioned for examination by electron microscopy. For this purpose, suspensions of the isolated particles were fixed in glutaraldehyde and immobilized in soft agar, and then small blocks of the agar (1 mm^2) were fixed in osmium tetroxide, imbedded in Epon-arldite and thin-sectioned [42]. Intact cotyledon tissue was also fixed, imbedded in plastic and thin-sectioned. Stained whole mounts and thin sections were examined with a Philips CM10.

Acknowledgement—The authors gratefully acknowledge financial support in the form of a grant-in-aid from the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

1. Brown, J. H., Paliyath, G. and Thompson, J. E. (1991) in *Plant Physiology – A Treatise. Growth and Development* Vol. X (Bidwell, R. G. S., ed.), pp. 227–275. Academic Press, New York.
2. Borochov, A., Halevy, A., Borochov, H. and Shinitzky, M. (1978) *Plant Physiol.* **61**, 812.
3. Suttle, J. E. and Kende, H. (1980) *Plant Physiol.* **65**, 1067.
4. Brown, J. H., Chambers, J. A. and Thompson, J. E. (1991) *Plant Physiol.* **95**, 909.
5. Draper, S. R. (1969) *Phytochemistry* **8**, 1641.
6. McKersie, B. D., Lepock, J. R., Kruuv, J. and Thompson, J. E. (1978) *Biochim. Biophys. Acta* **508**, 197.
7. Fobel, M., Lynch, D. V. and Thompson, J. E. (1987) *Plant Physiol.* **85**, 204.
8. Borochov, A., Halevy, A. H. and Shinitzky, M. (1976) *Nature* **263**, 158.
9. Paliyath, G. and Thompson, J. E. (1987) *Plant Physiol.* **83**, 63.
10. Brown, J. H., Paliyath, G. and Thompson, J. E. (1990) *J. Exp. Botany* **41**, 979.
11. Todd, J. F., Paliyath, G. and Thompson, J. E. (1990) *Plant Physiol.* **94**, 1225.
12. Serhan, C., Anderson, P., Goodman, P. and Weissman, G. (1981) *J. Biol. Chem.* **256**, 2736.
13. Seddon, J. M. (1990) *Biochim. Biophys. Acta* **1030**, 1.
14. Thompson, J. E. (1984) in *Physiology of Membrane Fluidity* (Shinitzky, M., ed.), pp. 85–108. CRC Press, Boca Raton, FL.
15. Garcia, R. E. and Mudd, J. B. (1978) *Plant Physiol.* **62**, 348.
16. Avato, P. (1984) *Planta* **162**, 484.
17. Kalinowska, M. and Wojciechowski, Z. A. (1983) *Phytochemistry* **22**, 59.
18. Kalinowska, M., Zimowski, J., Bucholc, M. and Wojciechowski, Z. A. (1989) *Phytochemistry* **28**, 2931.
19. Garcia, R. E. and Mudd, J. B. (1978) *Plant Physiol.* **61**, 357.
20. McKersie, B. D. and Thompson, J. E. (1979) *Biochim. Biophys. Acta* **550**, 48.
21. Platt-Aloia, K. A. and Thomson, W. W. (1985) *Planta* **163**, 360.
22. Paliyath, G. and Thompson, J. E. (1990) *New Phytol.* **114**, 555.
23. Barber, R. F. and Thompson, J. E. (1980) *J. Exp. Botany* **31**, 1305.

24. Goormaghtigh, E., Van Campenhoud, M. and Ruysschaert, J. M. (1981) *Biochem. Biophys. Res. Commun.* **101**, 1410.
25. Yao, K., Paliyath, G. and Thompson, J. E. (1991) *Plant Physiol.* **97**, 502.
26. Yao, K., Paliyath, G., Humphrey, R. W., Hallett, F. R. and Thompson, J. E. (1991) *Proc. Natl Acad. Sci. USA* **88**, 2269.
27. Yao, K., Wu, X., Thompson, J. E. and Carlson, J. E. (1993) *J. Cell Biochem.* **51**, 488.
28. Yao, K. and Thompson, J. E. (1993) *FEBS Letters* **323**, 99.
29. Yao, K., Paliyath, G. and Thompson, J. E. (1993) *J. Exp. Botany* **44**, 1207.
30. Murphy, D. J. (1990) *Prog. Lipid Res.* **29**, 299.
31. Greenwood, A. D., Leech, R. M. and Williams, J. P. (1963) *Biochim. Biophys. Acta* **78**, 148.
32. Hansmann, P. and Sitte, P. (1982) *Plant Cell Rep.* **1**, 111–114.
33. McRae, D. G., Chambers, J. A. and Thompson, J. E. (1985) *Biochim. Biophys. Acta* **810**, 200.
34. Steinmuller, S. and Tevini, M. (1985) *Planta* **163**, 201.
35. Ronning, C. M., Bouwkamp, J. C. and Solomos, T. (1991) *J. Exp. Botany* **42**, 235.
36. Torres, J. V. and Garcia-Olmedo, F. (1975) *Biochim. Biophys. Acta* **409**, 367.
37. Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911.
38. Morrison, W. R. and Smith, L. M. (1964) *J. Lipid Res.* **5**, 600.
39. Hamilton, J. G. and Comai, K. (1984) *J. Lipid Res.* **25**, 1142.
40. Grunwald, C. (1970) *Plant Physiol.* **45**, 663.
41. Hallett, F. R., Craig, T., Marsh, J. and Nickel, B. (1989) *Can. J. Spectros.* **34**, 63.
42. Glauert, A. M. (1965) in *Techniques for Electron Microscopy* (Kay, D. H., ed.), pp. 116–212. Blackwell, Oxford.