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Preservation and separation of endomembrane marker enzyme activity in potato leaf homogenates

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To minimize rapid browning and membrane degradation of crude microsomes, leaves of *Solanum tuberosum* (cv. Kennebec and cv. Katahdin) were initially homogenized in the presence of various inhibitors of polyphenol oxidase, phospholipase, and protease activity. To obtain and maintain marker enzyme activities used to identify plasma membranes, Golgi membranes, and endoplasmic reticulum, it was necessary to homogenize young leaves in the presence of sulfhydryls at pH 7.8. Further separation of these membranes, as determined by distribution of total activities of marker enzymes in linear sucrose density gradients, indicated a relatively pure plasma membrane fraction (1.15 g/cm³) free from contamination by thylakoids (1.19 g/cm³) and other endomembrane components. However, the distribution of specific activities across the gradient revealed that plasma membranes isolated from green tissue may be contaminated by Golgi membranes and not necessarily by plastid membranes.

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Afin de réduire au minimum le brunissement et la dégradation rapide des membranes de microsomes non purifiés, des feuilles de *Solanum tuberosum* (cv. Kennebec et cv. Katahdin) sont d'abord homogénéisées en présence de divers inhibiteurs de la polyphénol oxydase, de la phospholipase et de l'activité protéasique. Pour l'obtention et le maintien de l'activité des marqueurs enzymatiques utilisés dans l'identification des membranes plasmiques, des membranes de Golgi et du réticulum endoplasmique, il est nécessaire d'homogénéiser les jeunes feuilles en présence de sulfhydryles à pH 7,8. Une séparation additionnelle de ces membranes, telle que déterminée par la répartition des activités totales des marqueurs enzymatiques dans les gradients linéaires de sucrose révèle une fraction relativement pure de membrane plasmique (1,15 g/cm³) exempte de contamination par des thylakoïdes (1,19 g/cm³) et par d'autres composés endomembranaires. Cependant, la distribution des activités spécifiques à travers le gradient révèle que les membranes plasmiques isolées des tissus verts peuvent être contaminées par des membranes de Golgi et non pas nécessairement par des membranes plastidiales.

[Traduit par la revue]

Introduction

The isolation and purification of plasma membrane from green plant tissue has been difficult for at least two major reasons. First, the plasma membranes isolated in density gradients are likely to be contaminated by thylakoids (17). Second, chlorophyllous tissues, such as potato leaves, frequently brown upon tissue disruption since chloroplasts are a major component for polyphenol oxidase (PPO) activity (20). Products of the PPO reaction rapidly lead to browning and protein denaturation. Consequently, most subcellular fractionation work has been done with nongreen tissue to avoid these problems.

Because of the interest in studying host-pathogen interactions at the membrane level in leaf tissue, an attempt was made to isolate various organelles in potato leaf homogenates. Potato leaves are notorious for browning and endogenous membrane breakdown (8, 9), so an isolation procedure which minimized PPO activity and phospholipase activity was developed. This was necessary to preserve marker enzyme activity during long-term centrifugation when endoplasmic reticulum (ER), Golgi membranes, plasma membrane (PM), and thylakoids were further separated in linear sucrose density gradients.

Material and methods

Plant material

Solanum tuberosum (cv. Kennebec and cv. Katahdin) leaves were used throughout this study. Tubers were planted in soil and grown as described previously (9). Young leaves (1.5 to 2.0 cm long) or mature fully expanded leaves (5.5 to 7 cm) were cut at the base with stainless-steel scissors and placed in a prechilled mortar stored on ice. Whole

leaves were cut into small pieces in homogenization medium (standard medium contained 0.3 M sucrose, 5 mM EDTA, and 0.1 M HEPES-MES pH 7.8) and homogenized with a prechilled pestle. Various additives were used in the homogenization medium and are indicated in the text. The homogenate was passed through four layers of cheesecloth and immediately centrifuged.

Centrifugation

For differential centrifugation experiments, the crude homogenate was successively centrifuged at 1000 × g for 5 min, 6000 × g for 20 min, 13 000 × g for 15 min, and 100 000 × g for 35 min. The low-speed centrifugation was performed in a Sorvall SS34 rotor at 4°C and the 100 000 × g centrifugation step was done in a Ti 60 rotor with a Beckman L8-70 ultracentrifuge. For equilibrium density centrifugation, a 36-mL linear gradient (15 to 50% sucrose w/w) with 5 mM Tris-MES pH 7.8 was overlaid with 2 mL of the suspended 6000 to 100 000 × g pellet. Gradients were centrifuged in an SW 28 rotor at 4°C for 15 h at 84 000 × g and fractionated into 1.5-mL fractions.

Assays

K⁺-stimulated Mg²⁺-ATPase activity and detergent-activated uridine diphosphatase (UDPase) activity were determined as described previously (14). Cytochrome *c* oxidase (CCO) and antimycin A insensitive NADH cytochrome *c* reductase (NADH CCR) were assayed as described by Nagahashi and Baker (11). Glucan synthetase I (GS I) and glucan synthetase II (GS II) were assayed according to Ray (18) except for the following conditions. Incubation was at 30°C for 15 to 20 min and the assay volume was scaled up to accommodate 200 μL of each gradient fraction. Precipitation of membranes with 70% ethanol was enhanced by the addition of 2% BSA instead of boiled membranes. Chlorophyll was quantitated by the method of Arnon (2). Aliquots of crude membranes and gradient fractions were

TABLE 1. The specific activities of endomembrane marker enzymes in potato leaves. Marker enzymes associated with a microsomal fraction (13 000 to 80 000 × g pellet) isolated from young 'Kennebec' leaves (1.5 to 2.0 cm) were directly compared with those activities found in fully expanded mature leaves (5.5 to 7.0 cm). For both ages, 3 g fresh weight was homogenized

Leaf	K ⁺ -stimulated Mg ²⁺ -ATPase		Digitonin-stimulated Mn ²⁺ -UDPase		Antimycin A insensitive NADH CCR		Total protein, mg
	SA ^a	TA ^b	SA ^a	TA ^b	SA ^c	TA ^d	
Young leaves	5.05	1.53	25.35	7.68	0.366	0.111	0.303
Mature leaves	3.26	0.92	8.60	2.43	0.184	0.052	0.282

^aSpecific activity for phosphatases, $\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$.

^bTotal activity for phosphatases, $\mu\text{mol} \cdot \text{fraction}^{-1} \cdot \text{h}^{-1}$.

^cSpecific activity = $\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$.

^dTotal activity = $\mu\text{mol} \cdot \text{fraction}^{-1} \cdot \text{min}^{-1}$.

precipitated in 5% trichloroacetic acid and protein was estimated by a modified Lowry procedure (7).

Results

Preservation of marker enzyme activity

To prevent rapid browning of potato leaf homogenates, leaves were homogenized with various combinations of sulfhydryls (2-mercaptoethanol, 2-mercaptobenzothiazole, and dithiothreitol (DTT)) in the presence and absence of 2% BSA and (or) 1% insoluble polyvinyl pyrrolidone (10). Marker enzyme activity for PM (K⁺-stimulated ATPase), ER (antimycin A insensitive NADH CCR), and Golgi membranes (digitonin-stimulated UDPase) were compared with the specific activities of these markers in a microsomal fraction (13 000 to 80 000 × g) prepared in the standard homogenization medium. Highest specific activities for the PM marker and Golgi marker were found in the presence of DTT; however, the best activity for the ER marker was found when 2-mercaptoethanol or 2-mercaptobenzothiazole was included in the homogenization medium.

The addition of BSA, insoluble polyvinylpyrrolidone, or the protease inhibitor leupeptin (1) in the presence of sulfhydryls (data not shown) did not produce higher marker activity and they were not used in further experiments. Young leaves and fully expanded mature leaves were then homogenized in standard medium plus 5 mM DTT and 5 mM 2-mercaptoethanol. Specific activities for the endomembrane markers were considerably higher in young leaves compared with mature leaves (Table 1). When microsomes from young leaves were stored at 4°C in this homogenization medium, all marker enzyme activities were considerably diminished after 15 to 10 h. This observation can be explained by the fact that sulfhydryls also maintained 10 to 12 times higher phospholipase activity in 'Kennebec' leaf homogenates (8). When dibucaine was added to the homogenization medium to specifically inhibit the phospholipase (9), the anesthetic (2 mM) also inhibited the PM, Golgi, and ER markers by 40 to 50%. To preserve marker activity overnight, membrane fractions initially isolated in the presence of sulfhydryls were pelleted and suspended in standard homogenization medium (no sulfhydryls). These experiments clearly showed that certain reagents must be added to the standard homogenization medium to preserve marker activity, but the results further indicated that additives should not be used without checking all appropriate controls.

Differential centrifugation of the crude homogenate

Size separation by centrifugation showed that the organelle markers for mitochondria (CCO), microbodies (catalase), and chloroplasts (chlorophyll) were readily pelleted between 0 and 6000 × g (Table 2). Although this crude particulate fraction contained over 94% of the large organelles, substantial amounts of various endomembrane components were also sedimented at low centrifugal force (10). The crude membrane fraction pelleted between 6000 and 100 000 g contained 42 to 54% of the endomembrane components and also contained the highest specific activities for the PM, Golgi, and ER markers (Table 2). These results indicated that 6000 to 100 000 × g pellet was the best source of microsomes to be used for further purification.

Equilibrium density gradient centrifugation of microsomal markers

Distribution of total activities

To preserve marker enzyme activity during long-term density gradient centrifugation, the linear gradient was buffered at pH 7.8 in the absence of sulfhydryls to minimize phospholipase activity (8). The crude microsomal fraction (6000 to 100 000 × g pellet) was suspended in 2 mL of standard homogenization medium and not washed prior to overlaying on a gradient. After isopycnic centrifugation (Fig. 1), the ER marker formed a single peak at a density of 1.10 g/cm³, which was similar to the density of ER vesicles reported for other green (5, 13, 15) and nongreen (11, 16, 17, 18) plant tissues.

To determine the location of plasma membranes, the Mg²⁺-ATPase activity at pH 6.5 was measured in the presence and absence of 100 mM KCl (Fig. 1) and the K⁺-stimulated ATPase activity was used as a marker (17). In these experiments, it was not determined whether K⁺ or Cl⁻ stimulated the ATPase and hence the activity was referred to as KCl-stimulated ATPase. Fig. 1 shows four peaks of KCl-stimulated ATPase activity. The major peak of KCl-stimulated ATPase activity was found at 1.15 g/cm³ and was coincident with another frequently used PM marker (GS II). The GS II distribution was broad, but major peak coincided with the KCl-stimulated ATPase (Fig. 1). This ATPase activity was unlikely to be tonoplast in origin because of its density and insensitivity to nitrate ions (data not shown). A minor ATPase peak may be associated with tonoplast vesicles, which are typically found at 1.10 g/cm³, and a second minor peak was coincident with the Golgi marker at 1.12 g/cm³. A third minor peak was coinci-

TABLE 2. The distribution of organelle and microsomal markers in a potato leaf homogenate separated by differential centrifugation. Young potato leaves (8 g) were homogenized in 0.3 M sucrose containing 0.1 M HEPES-MES, pH 7.8, 5 mM dithiothreitol, and 5 mM 2-mercaptoethanol

Fraction	Organelle marker						Microsomal marker							
	Catalase		CCO		Chlorophyll		Total protein (mg)	% total protein	Antimycin A insensitive NADH CCR		K ⁺ -stimulated Mg ²⁺ -ATPase		Digitonin-stimulated Mn ²⁺ -UDPase	
	Specific activity	% total activity	Specific activity	% total activity	µg / mg protein	% total			Specific activity	% total activity	Specific activity	% total activity	Specific activity	% total activity
1 000 × g (5 min)	66.93	56.4	0.091	23.5	365	69.1	16.45	52.8	0.038	18.1	0.04	10.7	2.92	18.7
6 000 × g (20 min)	86.42	40.6	0.491	70.4	243	25.7	9.17	29.4	0.104	29.2	3.56	47.3	8.64	30.8
13 000 × g (15 min)	16.03	1.3	0.122	3.0	141	2.5	1.56	5.0	0.282	12.8	3.43	7.8	22.69	13.8
100 000 × g (30 min)	8.19	0.17	0.050	3.1	58	2.6	3.97	12.7	0.358	41.4	5.95	34.2	23.83	36.7

dent with thylakoid membranes at 1.19 g/cm³ but not with mitochondria, which equilibrated at 1.18 g/cm³ (data not shown).

The high amount of soluble UDPase activity at the top of the gradient was expected since a soluble enzyme has been reported in leaves from other species (4). To determine the location of latent UDPase activity (Golgi marker), the enzyme was assayed in the presence and absence of detergent (Fig. 1). Only the membrane-bound enzyme was activated by digitonin (1.12 g/cm³) so the digitonin-stimulated activity (14) could be distinguished from the soluble enzyme activity. The detergent-activated UDPase is considered to be a marker for Golgi membranes in nongreen tissue (14) and this is the first report confirming this observation in green tissue. GS I (another marker for Golgi membranes) was coincident with the digitonin-stimulated UDPase at 1.12 g/cm³; however, a second peak of GS I activity coincided with the plasma membrane marker (Fig. 1). The considerable overlap of GS I and GS II activity has been reported previously (18) and suggests that these markers have limited usefulness in certain tissues. Alternatively, the data could be interpreted to indicate that plasma membranes may be contaminated by Golgi membranes, although this conclusion would appear to be contradicted by the clear separation of other markers for Golgi membranes and PM (Fig. 1).

Similar separation of marker enzymes in linear sucrose gradients was achieved with microsomes isolated from young 'Katahdin' leaves (data not shown). With the exception of GS I activity, the PM isolated from both 'Kennebec' and 'Katahdin' leaves appeared to be separated from other endomembrane components and thylakoids.

Distribution of specific activities

In most cases where isopycnic separation of endomembrane components has been reported, the distribution of total activities of various markers were shown (3, 5, 11, 13, 15, 16, 18, 19, 21). To determine the degree of enrichment for any subcellular membrane in a sucrose gradient, the specific activity of a marker must be compared with that in the crude overlay (17). The distribution of the specific activities of the markers shown in Fig. 1 was calculated and shown in Fig. 2. The distribution of the KCl-stimulated ATPase showed one major peak of activity at 1.15 g/cm³ which was enriched 3.5-fold over the crude overlay. This observation indicated that the three minor peaks of KCl-stimulated ATPase shown in Fig. 1 may not have been assayed at their optimum conditions. It was likely that these other membrane-associated ATPases may have other cation requirements, different K_m values, and different pH optima than the PM-associated ATPase.

The ER marker also exhibited a single peak of activity and was enriched 2.6-fold over the crude fraction. This lower enrichment was probably due to the presence of soluble protein contaminants near the top of the gradient, as indicated by the protein profile (Fig. 2), which would effectively lower the specific activity of the ER marker. The distribution of the digitonin-stimulated UDPase activity in the gradient showed several interesting results. First, the specific activity of the Golgi marker (1.12 g/cm³) was enriched 3.8-fold over the crude overlay which is typical for membrane marker enrichment. Second, the peak of specific activity (fractions 9 and 10, Fig. 2) was shifted to a slightly higher density when compared with the distribution of total activity (fractions 8 and 9, Fig. 1). Third, the plasma membrane region in the gradient

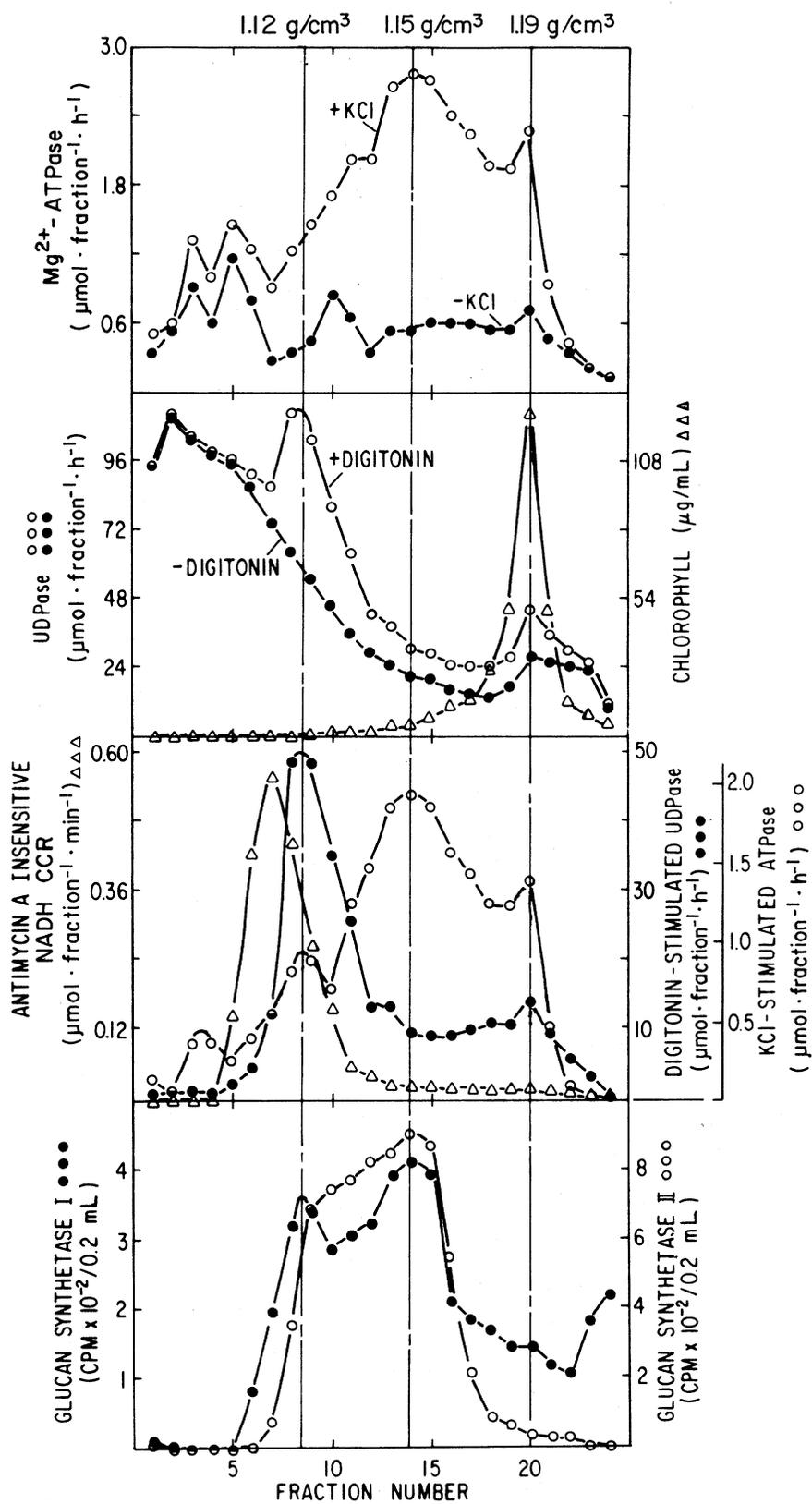


FIG. 1. Linear sucrose density gradient centrifugation of a crude microsomal fraction (6000 to 100 000 × g pellet) isolated from young potato leaves. The distribution of the total activities for endomembrane component markers and thylakoids is shown.

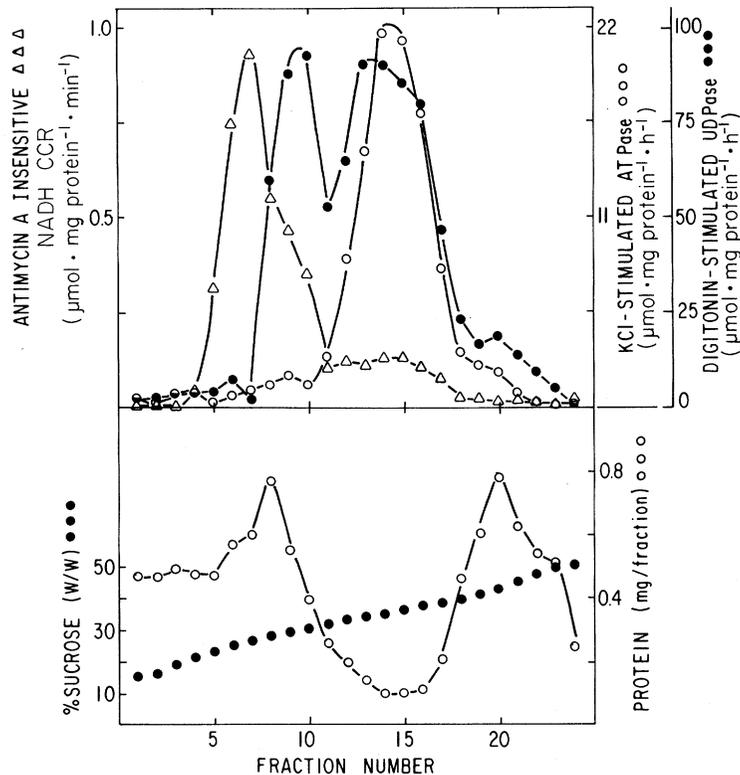


FIG. 2. Linear sucrose density gradient distribution of specific activities for endomembrane markers isolated from young potato leaves. The total activity per fraction shown in Fig. 1 was divided by the total protein per fraction to give specific activities.

was also enriched with Golgi membranes, as indicated by the presence of the digitonin-stimulated UDPase activity (Fig. 2), and this confirmed the distribution of GS I activity in Fig. 1. The fact that the plasma membrane was contaminated by detergent-stimulated UDPase activity was not apparent in Fig. 1 and was only revealed when the distribution of the specific activity of this Golgi marker was determined.

The two peaks of Golgi-associated marker activity were consistent with the two reported densities (1.12 and 1.15 g/cm³) of plant Golgi membranes (17). The lighter density membranes (1.12 g/cm³) may actually represent vesiculated Golgi membranes or resealed fragments of intact Golgi bodies. The evidence has been circumstantial; however, electron microscopy of the heavier fraction has shown intact Golgi bodies and intact cisternae (19), while micrographs of the lighter fraction indicated a vesicle population (12; G. Nagahashi and T. Seibles, unpublished results with Golgi membrane isolated from corn roots). If some of the intact Golgi bodies or cisternae are fragmented to a lighter density, this would imply that some of the regions or domains of the intact Golgi body must be more dense to give the overall density of 1.15 g/cm³. The presence of GS I activity at the bottom of the gradient (Fig. 1) supported this contention.

Discussion

An earlier report (16) alluded to the possibility that the density of plasma membranes isolated from members of the Leguminosae was lighter (1.13 to 1.15 g/cm³) than the density reported for members of the Gramineae (1.16 to 1.18 g/cm³). Although two reports (5, 6) have indicated that the density of PM from green tissue was 1.17 g/cm³, recent reports have also indicated a lighter density plasma membrane (1.13 to 1.15

g/cm³) from chlorophyllous tissue such as corn leaf protoplasts (15), pea cotyledons (13), and orchard grass (21).

The significance of the results reported here is several fold. Firstly, this work confirmed that green leaves from a nonleguminous plant may have a lighter density PM when compared with nongreen root tissue. Secondly, this report showed that plasma membrane isolated from 'Kennebec' leaves by conventional protocol has the same density as plasma membrane isolated from protoplasts from the same leaves (3). Apparently the digestion of cell walls from 'Kennebec' leaf cells did not affect the density of plasma membranes. The PM from protoplasts was identified by the coincidence of pH 6.5 Mg²⁺-ATPase activity and the diazotized sulfanilic acid surface label used to mark the protoplast cell membrane (3). The subcellular fractionation of potato leaf protoplasts did produce a major difference when compared with our conventional procedure. Thylakoids (1.16 g/cm³) were a major contaminant of PM isolated from protoplasts (3) but were not a major contaminant in our PM preparation from intact leaves (Fig. 1). The difference in thylakoid density may be age related since fully mature leaves were used in the protoplast study compared with young leaves used in this report. Furthermore, the homogenization medium composition was considerably different in the protoplast work (3).

Finally, it is significant and ironic that light-density PM isolated from green tissue may be contaminated by Golgi membranes and not by plastid membranes. Most subcellular fractionation studies involving the isolation of plasma membrane have been done on nongreen tissue to avoid cross contamination by thylakoids.

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