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STEROL BIOSYNTHETIC CAPABILITY OF PURIFIED MEMBRANE FRACTIONS FROM MAIZE COLEOPTILES

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Key Word Index—*Zea mays*; Gramineae; coleoptiles; cell fractionation; plasmalemma; NAA and NPA binding; cycloartenol-methyltransferase; cycloeucalenol–obtusifoliol isomerase; sterol glucosyltransferase.

Abstract—Membrane fractions were isolated from etiolated maize coleoptiles by differential and sucrose density gradient centrifugation. Specific membrane components were identified by using marker enzyme activities. Fractions were also tested for α-naphthylacetic acid (NAA) and *N*-naphthylphthalamic acid (NPA) binding activities. Evidence is presented for the isolation of a plasma-membrane fraction containing specific binding sites for NPA, a high concentration of sterols, and most of the total UDP-glucose sterol glucosyltransferase activity. A fraction rich in endoplasmic reticulum is shown to contain most of the binding sites for NAA and all of the activity of both *S*-adenosyl-L-methionine-Δ24 cycloartenol methyltransferase and cycloeucalenol-obtusifoliol isomerase.

INTRODUCTION

In higher plants free sterols and sterol esters commonly occur together with sterol glucosides (SG) and their acylated derivatives (ASG) [1]. Free sterols, SG and ASG are constituents of plant membranes [2]. We wanted to study the biogenesis of the endomembranes of higher plants by using sterols as markers. One approach is to consider the subcellular location of the pathways involved in sterol biosynthesis. The enzymes involved in the synthesis of squalene precursors are soluble [3]. The conversion of squalene to sterols is catalysed by membrane-associated enzymes, but its exact location in cells of higher plants has not yet been determined accurately. In mammals, cholesterol biosynthetic enzymes are bound to membranes of smooth and rough endoplasmic reticulum (ER) [4]. In yeast, the *S*-adenosyl-L-methionine (SAM)-Δ24 sterol methyltransferase is reported to be of mitochondrial origin [5]. In higher plants, sterol biosynthesis is characterized by specific features such as cyclization of squalene 2,3-epoxide to produce cycloartenol, opening of the cyclopropane ring, and double alkylation at C-24 [6]. Thus the presence in higher plant cells of sterol biosynthetic enzymes that do not exist in animals and fungi suggest that there are interesting differences in the respective enzyme location in membranes or organelles. We have previously shown that in etiolated bean leaves, farnesyl pyrophosphate squalene synthetase is associated mainly with smooth and rough vesicles of the microsomal fraction [7] and that in etiolated maize coleoptiles, the activities of the SAM-Δ24 cycloartenol methyltransferase (CMT) and UDP-glucose sterol β-glucosyltransferase (UDP-GlcST) were associated respectively with membrane fractions rich in ER and in plasma membranes [8]. These fractions were obtained from a 100000 *g* pellet after discontinuous sucrose gradient centrifugation. We thought that sedimenting microsomal membranes by centrifugation and resuspending microsomal pellets before gradient centrifugation might change the size of membrane vesicles (possibly by fusion), resulting in poor vesicle separation. To avoid this possibility and to increase the purity of the separated membranes, we have investigated an alternative procedure consisting in layering of a post-mitochondrial supernatant onto a modified sucrose gradient. Results reported in this paper confirm earlier data [8, 9] and show the clear separation of two classes of membrane vesicles, one involved in free sterol biosynthesis and the other in free sterol glucosylation.

RESULTS

Membrane fractions from coleoptiles of etiolated maize seedlings were characterized following differential and equilibrium density gradient centrifugation. They were identified by assays for established marker enzymes: ER, NADPH- or NADH-cyt *c* reductase [10] and cinnamic acid-4-hydroxylase (CAH) [11, 12]; Golgi apparatus, β-glucan synthetase of high affinity for UDP-Glc (GS I) [13, 14]; plasmalemma, β-glucan synthetase of low affinity for UDP-Glc (GS II) [13]. Succinate cyt *c* reductase activity was used as marker for inner mitochondrial membranes. Fractions were also tested for NAA and NPA binding, sterol contents, and the activities of enzymes involved in sterol biosynthesis: CMT, cycloeucalenol–obtusifoliol isomerase (COI) and UDP-Glc ST.

Distribution of enzyme activities and sterols in crude fractions

Most of the total activities of NADPH-cyt *c* reductase, CAH and GS II was found in the microsomal fraction C_{100} (Table 1). NAA and NPA specific binding activities were essentially associated with the 6000 *g* supernatant, which contained 60 to 80% of the auxin binding sites (Table 1). The microsomal fraction was richest in free

Table 1. Distribution of enzyme activities and sterols in crude fractions from etiolated maize coleoptiles following differential centrifugation

Centrifugation fraction	c_1		c_6		c_{100}	
	s_A	t_A	s_A	t_A	s_A	t_A
Succinate cyt *c* reductase	0.06	1.8	0.42	20.2	0	0
NADPH cyt *c* reductase	0.2	4.7	0.5	21	1.3	33
Cinnamate hydroxylase	0.8	30	0.6	40	11.6	745
GS II	4.4	130	4.7	230	8.8	345
NPA binding	—	—	—	70	—	252
NAA binding	—	—	—	13.8	—	356
Sterols	0.1	3.6	0.3	10	0.7	26

c_1, c_6 and c_{100}. pellets from respectively 1000 *g*, 6000 *g* and 100000 *g* centrifugations. t_A: total activity; s_A: specific activity on a per mg protein basis. Succinate and NADPH cyt *c* reductase activities are expressed as nmol cyt *c* reduced $\times 10^{-2}$/min; CAH and GS II activities as nmoles of product formed/hr; NPA and NAA binding activities as cpm $\times 10^{-3}$; sterols as $\mu g \times 10^{-2}$. NPA and NAA bindings were measured using a 6000 *g* supernatant.

sterols. Succinate-cyt *c* reductase was found only in the 6000 *g* pellet, which contained most of the mitochondria. Table 2 shows that all three enzymes involved in sterol biosynthesis that we studied were found mainly in the microsomal fraction.

To separate the various components of the microsomal fraction, the 6000 *g* supernatant was applied to sucrose gradients prepared as described in the Experimental. In most experiments, membranes of this supernatant were first concentrated by centrifugation onto a 60% (w/v) sucrose cushion. After equilibrium density gradient centrifugation, fractions were collected and assayed for absorbancy at 280 nm (A_{280}), for sucrose percentages and for the various enzyme activities described above.

Distribution of marker enzyme activities after sucrose density gradient centrifugation

Two major membrane fractions were recovered in the gradient as discrete and well separated protein bands, a light fraction at the 20 to 30% sucrose step and a heavy one at the 45 to 60% sucrose interface (Fig. 1a). The NADH-cyt *c* reductase and CAH activities showed a single distinctive peak at the 20 to 30% step, providing evidence that the membranes in this fraction were in part derived from the ER (Fig. 1b). In the conditions of

Table 2. CMT, COI and UDP-Glc ST activities in crude fractions from etiolated maize coleoptiles following differential centrifugation

Centrifugation fraction	C_1		C_6		C_{100}	
	s_A	t_A	s_A	t_A	s_A	t_A
CMT	0.2	9.5	0.8	29	2.6	136
COI	0.4	11.5	1.5	57	9.5	248
UDP-Glc ST	5.0	60.5	4.5	148	9.1	307

C_1, C_6, C_{100}: pellets from respectively 1000 *g*, 6000 *g* and 100000 *g* centrifugations. t_A: total activity per fraction; s_A: specific activity on a per mg protein basis. All activities are expressed as nmol product formed/hr

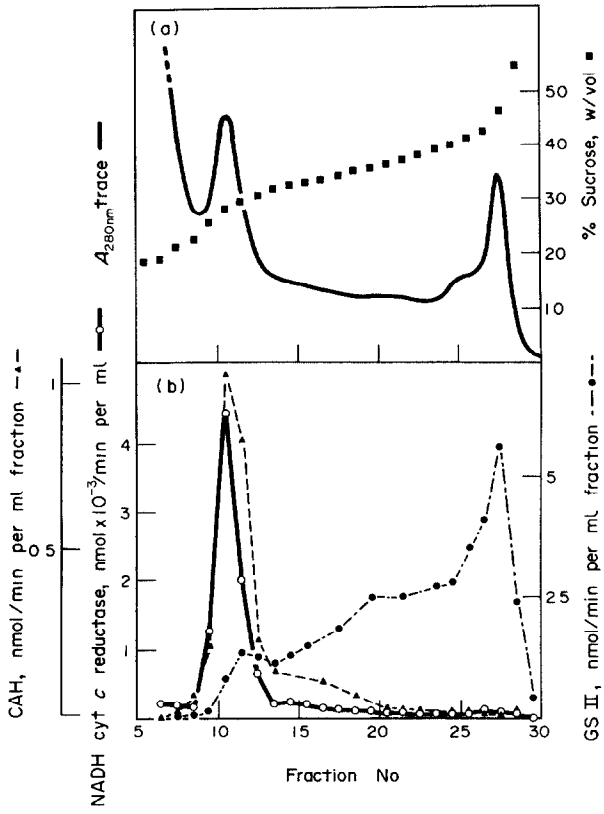

Fig. 1. Distribution of NADH cyt *c* reductase, CAH and GS II in gradient fractions. 70g coleoptiles from etiolated maize seedlings were homogenized in 70 ml extraction buffer. The homogenate was centrifuged at 1000 *g* for 5 min and the supernatant at 6000 *g* for 10 min to sediment mitochondria. The resulting supernatant was centrifuged on a 60% (w/v) sucrose cushion at 110000 *g* for 45 min in a Beckman SW 27 rotor. The membranes at the interface were collected, diluted with Tris buffer and layered on top of 20% sucrose (4 ml) resting on a 30 to 45% linear sucrose gradient (22 ml) and 60% sucrose (4 ml). The gradient was centrifuged at 110000 *g* for 4 hr (rotor SW 27). Fractions of 1.2 ml were collected.

homogenization used, ribosomes were dissociated from rough ER, with resulting sedimentation of membranes at low density. It can be seen (Fig. 1b) that using a UDP-Glc concentration of 2.9 mM, the most active synthesis of β-glucans was in membranes at the 45 to 60% sucrose interface. Some activity was also detected in the light fraction. In order to determine the distribution of Golgi vesicles in the gradient, fractions were tested for inosine diphosphatase and GS I activities which have been described as being associated with plant dictyosome membranes [13, 14]. Latent inosine diphosphatase activity was broadly distributed in the sucrose gradient with no distinct peak (data not shown here). In Fig. 2 are reported results from another gradient, showing the distribution of the two glucan synthetase activities at low (GS I) and high (GS II) concentration of substrate. These two enzyme activities were clearly separated one from another, the GS I banding at a lower density than the GS II.

NAA and NPA binding

We have previously demonstrated specific interactions

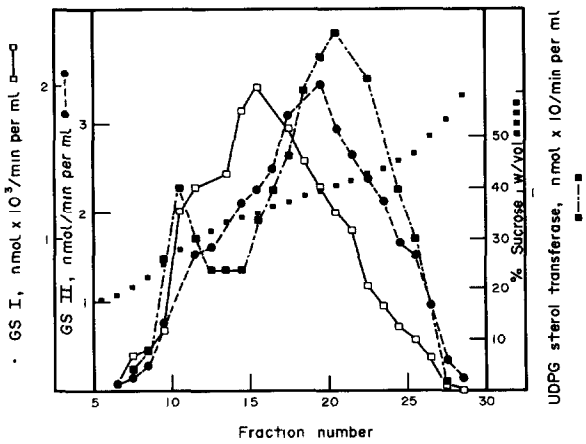

Fig. 2. Distribution of GS I, GS II and UDP-Glc ST activities in gradient fractions. The gradient used in this experiment consisted of 20% sucrose (4 ml), a 30 to 50% linear sucrose gradient (22 ml) and 60% sucrose (4 ml). It was centrifuged as described in Fig. 1.

of NAA and NPA with membrane receptors [15]. These interactions were shown to be specific, reversible and saturable [16, 17]. As this binding activity was shown to be essentially associated with a 6000 *g* supernatant (Table 1), we tested NPA and NAA binding activities with all the fractions from the gradient (Fig. 3). NAA-specific binding occurred exclusively in membranes at the 20 to 30% sucrose interface. No significant binding of NAA was detected in the heavy fraction (45 to 60% sucrose interface). NPA-specific binding showed a distinctive peak corresponding to the heavy fraction; little specific binding was detected in the light fraction.

Sterol analysis

Analysis concerned exclusively the free form of sterols (Table 3). The same relative sterol distribution was found in all the fractions (stigmasterol 56%, sitosterol 24%, campesterol 10%, unidentified sterols 9%, isofucosterol traces, cholesterol traces). In contrast, concentrations of sterols in different membrane systems differed profoundly: the heavy fraction contained much more sterols than the light fraction and showed a marked enrichment in comparison with crude fractions (Table 1). Purified mitochondria had a much lower concentration of sterols. Differences were even greater when sterol : phospholipid molar ratios were considered. This ratio was 5 times as high in the heavy fraction as in the light fraction.

Distribution of CMT, COI and UDP-Glc ST activities (Fig. 4)

Synthesis of 24-methylenecycloartanol from cycloartenol and SAM-[Me-^{14}C] was found only in the light fraction. A similar distribution of the COI activity was observed. In contrast, most of the total UDP-Glc ST activity was bound with the heavy fraction, although some was detected in the light fraction. As shown in Fig. 2, the distribution of UDP-Glc ST activity closely correlated with that of GS II activity and was clearly separated from that of GS I activity.

Comparison of the distribution of UDP-Glc ST and UDP-galactose diglyceride transferase (UDP-Gal DT)

Gradient fractions corresponding to the light fraction were pooled and centrifuged. The same was done with heavy fraction and mitochondria. UDP-Glc ST and UDP-Gal DT activities were assayed in all three pellets. UDP-Glc ST transferred glucose into endogenous sterol acceptors to give SG and ASG; UDP-Gal DT transferred galactose into endogenous diglyceride acceptors to give monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG). Negligible UDP-Glc – UDP-Gal epimerase was found in all these fractions. UDP-Glc ST was associated mainly with the heavy fraction, with low activities in the light fraction and in the mitochondria. UDP-Gal DT was present mainly in the light fraction, while the heavy fraction and the mitochondria had negligible activities (Table 3).

DISCUSSION

From a post-mitochondrial supernatant of etiolated maize coleoptiles, we isolated two major membrane fractions as well separated protein bands, a light fraction

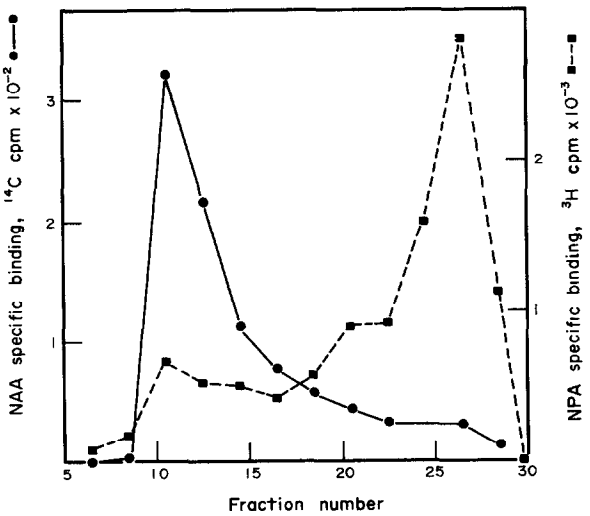

Fig. 3. Distribution of NAA and NPA specific binding in gradient fractions obtained as in Fig. 1.

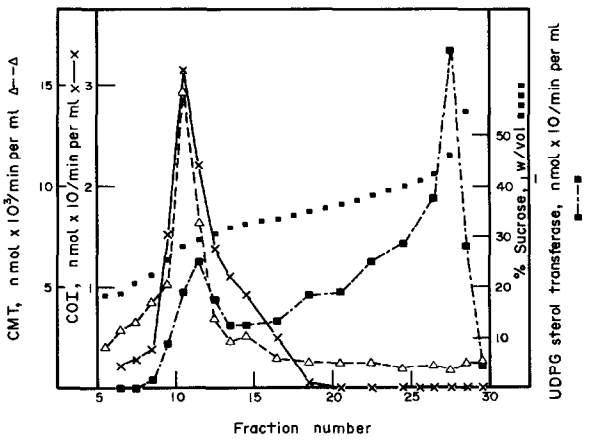

Fig. 4. Distribution of CMT, COI and UDP-Glc ST activities in gradient fractions obtained as in Fig. 1.

Table 3. Sterols and UDP-Glc ST and UDP-Gal DT activities in membrane fractions from etiolated maize coleoptiles

	Light fraction	Heavy fraction	Mitochondria
Sterols* μg/mg protein	80 ± 10	200 ± 20	25 ± 5
sterols/phospholipids molar ratio	0.20	1.0	0.20
UDP-Glc ST nmol product/hr/mg protein	SG 14 ASG 7	SG 103 ASG 120	SG 19 ASG 7.5
UDP-Gal DT pmol product/hr/mg protein	MGDG 57 DGDG 33	MGDG 3.5 DGDG 3.5	MGDG 3.5 DGDG 1.5

* Stigmasterol 56%, sitosterol 24%, campesterol 10%, unidentified sterols 9%, isofucosterol and cholesterol traces. Gradient fractions were obtained as in Fig. 1. Fractions corresponding to the light and heavy fractions were pooled, pelleted and resuspended in Tris buffer. Mitochondria were prepared as described in Experimental.

at the 20 to 30% sucrose step ($d \sim 1{,}12$) and a heavy one at the 45 to 60% sucrose interface ($d \sim 1{,}17$). As no succinate-cyt *c* reductase was detected in the material submitted to the gradient, mitochondrial internal membrane contamination in the two fractions was probably negligible. The recovery of total NADH-cyt *c* reductase and CAH in the light fraction provided evidence that it was rich in ER. This fraction also contained most of the UDP-Gal DT activity, which may reflect some contamination with amyloplast envelopes [18]. As no reductase or CAH activity was detected in the heavy fraction, we conclude that the latter was not contaminated by vesicles originating from the ER. The distribution in the gradient of GS II showed a major peak of activity associated with the heavy fraction, suggesting that the latter consisted mostly of vesicles derived from the plasma membrane. In onion stem, at concentration of 1 mM UDP-Glc, β-1,3 glucans have been shown to be synthesized by both dictyosomes and plasma membranes [13]. Thus part of the β-glucan synthetase activity appearing in the heavy fraction might originate in Golgi vesicles. To ascertain whether this was the case, GS I, a suggested marker for Golgi membranes [13, 14], was measured in each fraction of the gradient; the peak for GS I activity is clearly distinct from the peak for GS II (Fig. 2), suggesting that Golgi vesicles would be separated from plasma membranes in our experimental conditions. This conclusion is in agreement with recent results of Ray *et al.* [19] obtained with the same material. Free sterols (mainly stigmasterol, sitosterol, and campesterol) are present in all membrane fractions. Whereas the relative distribution of these three sterols did not differ significantly from one fraction to another, their concentrations in membranes changed profoundly: sterols were present in only moderate amounts in the light fraction but, in contrast, were contained in remarkably high concentrations in the heavy fraction. Like plasma membrane of animal cells, the plant plasmalemma should be characterized by a high content of free sterols and a high sterol: phospholipid molar ratio. Hodges *et al.* [20] have presented evidence for a K^+-dependent ATPase activity associated with a high content of sterols in a plasma-membrane-enriched fraction from oat roots, that had the same density as our fraction.

In contrast, purified mitochondria contained very little sterols. This result disagrees with data obtained previously in our laboratory with the same material [9] and in another laboratory [21]. The high concentrations of sterols found previously in mitochondria could be explained by contamination with plasma membranes whose density is close to that of mitochondria [22]. The use in the present work of a velocity gradient rather than an isopycnic gradient probably allowed us to separate these two types of membrane. It is also possible that the mitochondrial fraction considered in that work consisted of mitochondria partly stripped of their external membrane, which is the part richest in sterols [23]. High-affinity ($K_d = 10^{-6}$M) NAA receptors were shown to be associated with the ER- rich fraction only. Very low binding was detected in the heavy fraction, suggesting that plasma membranes would contain either only a few high-affinity sites for NAA recognition or a larger number of low-affinity sites. These results are consistent with recent data [19, 24] but differ from others [25] showing that appreciable NAA receptors would be associated with a plasma-membrane-rich fraction. The isopycnic distribution of high-affinity ($K_d = 10^{-7}$M) NPA receptors essentially correlates with that of GS II, confirming that NPA might be a marker for plasma membranes, as suggested before [26]. The physiological meaning of the binding of NAA is debatable. The specific binding detected in maize coleoptiles has been considered as a primary event in the mechanism of action of auxinic compounds during cell elongation, as discussed elsewhere [16, 27, 28]. As the cell elongates, the total surface of the plasma membrane and of the cell wall increases. Thus, one of the primary events occurring after auxin binding could be a synthesis of new membranes and/or a rearrangement of preexisting ones [29]. Therefore an early effect of auxins on membrane lipid biosynthesis and/or rearrangement may be expected. Sterols are likely candidates for such an auxin effect since they play a considerable role in controlling membrane fluidity by their interaction with phospholipids [30]. A prerequisite for clarification of the role of sterols during IAA-induced cell enlargement was to determine the cellular sites of sterol biosynthesis. We have shown here that CMT and COI, two enzymes involved in sterol biosynthesis in higher plants, were associated with the light fraction of the gradient and correlated with NADH-cyt *c* reductase and CAH activities and with NAA binding sites. Thus these two enzymes are located in the ER, in agreement with previous results showing that farnesyl pyrophosphate-squalene synthetase was associated with smooth and rough ER in etiolated bean leaves [7]. In contrast, the UDP-Glc ST activity was shown to be located in a fraction rich in GS II activity and NPA-specific binding sites which are both considered to be markers for plasma membrane; it did not correlate with the activity of GS I, a presumed marker for Golgi apparatus (Fig. 2). Thus our results strongly suggest that glycosylation of sterols occurs in plasma membranes. This hypothesis is attractive because plasma membrane also contained the highest concentration of free sterols (Table 3), which could serve as substrates for the synthesis of steryl glucosides. Our results are not in agreement with those of other authors, who recently provided evidence that the main site of sterol glucosylation is on Golgi-vesicle-rich fractions from onion stem [31], *Calendula officinalis* seedlings [32], and *Phaseolus aureus* hypocotyls [33]. In contrast, our results agree with those of Van der Woude *et al.* [13], which showed an association

of glycolipid synthetases with plasma membranes from onion stem and a more limited capability for glycolipid synthesis by dictyosome membranes.

In any case, our results as well as those of the authors cited above clearly indicate that the free sterols and the glucosylated forms are synthesized by enzymes located on different cellular membranes. Free sterols are synthesized on the ER membranes, into which they may then be integrated. According to the membrane-flow theory [34] or by some other mechanism, they would appear in other components of the endomembrane system; then, depending perhaps on the plant studied, they could be glucosylated to SG either in the Golgi apparatus as postulated by Bowles [33] or in plasma membranes as shown in the present work; SG are finally acylated to yield ASG [1]. SG would behave very differently from free sterols in membranes as they are more hydrophilic; such a hypothesis is in agreement with the experiments of Grunwald [35]. Thus sterol glucosylation in modifying the lipid bilayer of membranes might play an important role in regulating some membrane activities, especially those involved in cell enlargement [27].

EXPERIMENTAL

Radiochemicals. NAA-[1-^{14}C] (44 mCi/mmol), UDP-Glc-[U-^{14}C] (312 mCi/mmol), UDP-Gal-[U-^{14}C] (321 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.). NPA-[^{3}H] (1 Ci/mmol) and SAM-[Me-^{14}C] (46 mCi/mmol) were supplied by the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France). Unlabelled NAA was from Sigma Chemical Co. (St Louis, Mo., U.S.A.). NPA was prepared as described previsouly [15].

Plant material. Maize seeds (*Zea mays* cv INRA 258), were allowed to germinate on moist vermiculite in the dark at 26°. The coleoptiles were excised after 6 days of germination.

Isolation of membranes. Maize coleoptiles were ground in a chilled mortar at 4° in the presence of 0.5 M mannitol, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.5% BSA and 0.1 M Tris–HCl, pH 8. One ml of medium was used per g fr. wt of coleoptiles. In the case of NAA and NPA binding assays, no mercaptoethanol was added in the grinding medium. The homogenate (pH ~ 7.5) was filtered through 4 layers of cheesecloth and successively centrifuged at 1000 *g* for 5 min to remove cell debris and starch and at 6000 *g* for 10 min to sediment mitochondria. The resulting pellet was resuspended in 20 mM Tris–HCl (pH 7.4) containing 0.5 M mannitol, 1 mM EDTA and 0.2% BSA. The mitochondria were layered onto a discontinuous sucrose gradient consisting of 9 ml each of 60, 45, 30 and 20% (w/v) sucrose soln in 20 mM Tris–HCl (pH 7.4). The gradient was centrifuged at 40500 *g* for 1 hr (Beckman rotor SW 27). Purified mitochondria were collected at the 45 to 60% sucrose interface. They were then diluted with 20 mM Tris–HCl and pelleted by centrifugation at 40000 *g* for 30 min (rotor 60 Ti). The 6000 *g* supernatant was layered onto a gradient consisting of a 4 ml cushion of 60% sucrose (w/v), 22 ml of soln increasing linearly in concn from 30 to 45% sucrose, and a 4 ml layer of 20% sucrose. All sucrose solns were prepared in 20 mM Tris–HCl (pH 7.5) and 2.5 mM mercaptoethanol. The gradient was centrifuged at 110000 *g* for 4 hr (Beckman rotor SW 27). Another sucrose gradient was also used. It consisted of a linear part from 30 to 50% sucrose rather than 30 to 45%. It was centrifuged in the same conditions. When larger amounts of material were necessary, the membranes of the 6000 *g* supernatant were first concd onto a 60% sucrose cushion by centrifugation at 110000 *g* for 45 min. The membrane fraction at the interface was collected, diluted with 0.1 M Tris–HCl (pH 7.5) and 10 mM mercaptoethanol, and applied on the sucrose gradients described above. After centrifugation of gradients, 1.2 ml fractions were collected using a density gradient fractionator (ISCO, Model 640). Then the sucrose percentages and enzyme activities of the different fractions were measured.

Enzyme assays. NADPH-, NADH- and succinate-cyt c reductase were assayed by following the reduction of cyt *c* at 550 nm as described in ref. [36]. *CAH activity* was measured according to ref. [11]. *Activities of GS I and GS II* were determined by measuring the incorporation of UDP-Glc-[U-^{14}C] into 70% EtOH-insoluble material following [14]. The reaction mixture of GS I assay consisted of 100 µl of gradient fraction, 16 mM $MgCl_2$ and 0.04 µCi (0.9 µM) UDP-Glc-[U-^{14}C] in 0.1 M Tris–HCl pH 8. This mixture was incubated at 30° for 5 min. The reaction mixture of GS II assay contained 50 µl of gradient fraction, 0.02 µCi (2.9 mM) UDP-Glc-[U-^{14}C] in 0.1 M Tris–HCl pH 8 and was incubated at 30° for 15 min. The incubations of two GS assays were stopped by adding 2 ml 70% EtOH containing 20 mM $MgCl_2$ and 20 µl of crude mitochondrial suspension (to obtain a sizeable precipitate) and then by heating for 1 min in boiling water. Insoluble material was washed ×3 with 2 ml of 70% EtOH and centrifuged at 1000 *g* for 10 min. In the case of GS I, the pellets were extracted once more with 1 ml CH_2Cl_2–MeOH(2:1). In all cases, washed pellets were then transferred to vials with 1.5 ml of Triton X100 1% in EtOH and counted in Bray soln.

Inosine diphosphatase was assayed at pH 7.4 with 3 mM IDP and 2 mM $MgCl_2$ at 30° for 30 min. Proteins were pptd with 10% TCA and release of Pi was measured according to the method of ref. [37].

UDP-Gal DT was assayed by adding 0.1 µCi UDP-Gal-[U-^{14}C] to 200 µl of membrane suspensions obtained after pooling and pelleting gradient fractions. The total mixture was incubated at 30° for 1 hr and was then brought to a total vol. of 1 ml with 0.1 M Tris–HCl (pH 8). After heating for 1 min in boiling H_2O, the suspension was extracted with 6 ml CH_2Cl_2–MeOH (2:1). The organic layer was washed with 2 ml MeOH–H_2O (1:1). Glycolipids were chromatographed in Me_2CO–C_6H_6–H_2O (45:15:2). Radioactive MGDG and DGDG were eluted with CH_2Cl_2–MeOH (4:1) and counted in Bray soln.

NAA and NPA binding assays were performed as in ref. [15] with the following modifications: fractions from the gradient were diluted with 1 vol. of 20 mM citrate buffer containing 10 mM $MgSO_4$, pH 5.5. Each incubation was carried out in duplicate, one containing the membrane suspension and a small concn of the radioactive ligand (0.1 µCi = 5×10^{-8} M in the case of NPA; 0.005 µCi = 5×10^{-8} M in the case of NAA), the other containing, in addition to the same components, a saturating concn of unlabelled ligand (5×10^{-4} M). After 30 min of incubation at 0°, the incubates were centrifuged at 55000 *g* for 30 min. The supernatant was discarded, and the bottom of the tube was cut off and transferred into scintillation vials containing 1 ml EtOH. After 4 hr, the scintillation cocktail (Bray) was added and the vials were counted.

SAM-Δ24 cycloartenol methyltransferase (CMT). The incubation mixture contained 0.9 ml of freshly prepd gradient fraction, 100 µM of cycloartenol, and 100 µM of SAM-[Me-^{14}C] (0.3 µCi) in a total vol. of one ml. The sterol substrate was prepd by sonication of the cycloartenol suspension in aq. 1% Tween 80 immediately before use. The incubation mixture was maintained at 30° for 1 hr and the reaction was terminated by the addition of an equal vol. of KOH in EtOH. Samples were then extracted ×3 with 3 vol. of petrol and assayed for radioactivity. We have previously shown that radioactivity was incorporated into 24-methylenecycloartenol [38].

Cycloeucalenol–obtusifoliol isomerase(COI). The reaction mixture contained 0.9 ml of gradient fraction and 50 µM of cycloeucalenol in a total vol. of 1 ml. Incubations were carried out at 30° for 1 hr and stopped by the addition of 1 vol. of KOH in EtOH. The mixtures were extracted ×3 with 3 vol. of petrol and the extract was then submitted to TLC, using CH_2Cl_2 as developing solvent (2 runs). The 4α-methylsterols were eluted and analysed by GLC on a 1% SE-30 column at 240°. Quantitative determinations were made by measured peak areas of cycloeucalenol and obtusifoliol.

UDP-Glc sterol glucosyltransferase (UDP-Glc ST). Reaction

mixtures contained 0.5 ml of gradient fraction, 0.3 mM UDP glucose-[U-^{14}C] (0.1 μCi), 0.2 mM cholesterol combined with 0.08% Triton X100 and 8 mM $MgCl_2$. The mixtures were brought to a total vol. of 1 ml with 0.1 M Tris-HCl (pH 8). The reaction was run at 30° and stopped after 30 min by heating for 1 min in boiling H_2O. After cooling, the mixture was extracted with 6 ml CH_2Cl_2–MeOH (2:1). The aq. phase was discarded and the organic layer was washed with 2 ml MeOH–H_2O (1:1) to remove traces from radioactive aq. products, and evapd to dryness. The ^{14}C radioactivity incorporated into total glycolipids was counted in a liquid scintillation spectrometer. In some assays, glycolipids were chromatographed in CH_2Cl_2–MeOH–H_2O (170:30:1). SG and ASG were isolated by elution with CH_2Cl_2–MeOH (4:1) and counted for radioactivity. No trace of radioactivity was detected in galactolipids MGDG and DGDG.

Other assays. Sucrose concentrations were determined refractometrically. *Protein* was measured according the method of Lowry using BSA as the standard. *Sterol analysis.* The fractions were extracted ×3 with 3 vol. of petrol and the extract then chromatographed using CH_2Cl_2 as developing solvent (2 runs). The 4-demethylsterols were eluted and determined quantitatively as their acetates by GLC. The carrier gas was N_2 (30 ml/min) and detection was by FID. A glass column packed with 1% SE-30 was used at 240°. Quantitative determinations were made by measured peak area compared to that of an int. stand. of cholesterol (which is present in only trace amounts in our membrane fractions). Phospholipid analysis: phospholipids were extracted ×2 with 6 vol. of $CHCl_3$–MeOH (2:1). The lower organic phase was removed and evapd to dryness. Then 70% perchloric acid (0.9 ml) and 1 drop of 2.5% ammonium molybdate were added. The mineralization was performed for 15 min until total decoloration. Bidistilled H_2O (7 ml) was added and the mixture heated for 10 min in boiling H_2O. Then 2.5% ammonium molybdate (1.5 ml) and 10% ascorbic acid (1.5 ml) were added. The mixture was finally incubated at 37° for 2 hr and OD measured at 820 nm. Appropriate amounts of standard solns were assayed in the same conditions.

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