

Lipid Composition of Organelles from Germinating Castor Bean Endosperm¹

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ABSTRACT

Glyoxysome, endoplasmic reticulum, mitochondria, and proplastid fractions were isolated from endosperm of castor beans (*Ricinus communis*) germinated for 5 days at 30 C. Samples from sucrose density gradients were diluted with 0.15 M KCl and the membranes pelleted. Lipid extracts of these membranes were analyzed for phosphoglyceride, acyl lipid, and sterol content. The endoplasmic reticulum contains 1.24 μmol of phosphoglyceride per mg of protein; the mitochondria, 0.65 $\mu\text{mol}/\text{mg}$; and the glyoxysome membranes, 0.55 $\mu\text{mol}/\text{mg}$. Phosphatidyl choline and phosphatidyl ethanolamine are the most abundant lipids in all membranes studied, accounting for 70% or more of the lipid phosphorus and 50% or more of the fatty acid. Glyoxysome membranes and endoplasmic reticulum also contain phosphatidyl inositol (respectively, 9 and 17% of the lipid phosphorus) and free fatty acids (13% of the total fatty acid in each). Compared with other organelles, mitochondrial membranes have more phosphatidyl ethanolamine relative to phosphatidyl choline and are characterized by the presence of cardiolipin, in which 80% of the fatty acid is linoleate. The relative amounts of linoleate, palmitate, oleate, stearate, and linolenate in each of the phosphoglycerides are constant regardless of the membrane source. Stigmasterol and β -sitosterol are present in the membranes (1-9 nmol each/mg protein).

The data provide further evidence that glyoxysome membranes are derived from the endoplasmic reticulum but at the same time indicate some differentiation.

Virtually all of the lipid in seeds of castor bean (22) is the triglyceride of ricinoleic acid, a monounsaturated hydroxylated fatty acid not found in membrane phospholipids. It is stored in the endosperm tissue in lipid bodies or spherosomes. During germination of the seed, the lipid bodies disappear and three important membranous organelles arise (29) which participate in the conversion of the stored tricinolein to sucrose (3). These organelles, the glyoxysomes, endoplasmic reticulum and mitochondria have been separated on sucrose density gradients and their enzymic components well characterized. The glyoxysomes contain all of the enzymes of β oxidation and of the glyoxylate cycle necessary to produce succinate from fatty acids (3). The ER² is responsible for the synthesis of membrane phospholipids of the glyoxysomes and mitochondria (3, 15, 20, 21, 24).

The glyoxysome membrane is a single lipid bilayer, presumably permeable to sucrose and metabolites such as succinate. The membrane has been isolated and some of its protein components characterized (13). The phospholipid components of

whole glyoxysomes have been described briefly (8, 9). Since the glyoxysome apparently lacks the ability to synthesize its lipid components (3, 24), its lipid composition is here compared to the apparent synthetic source, the ER, and to other organelles of the germinating castor bean. From this material it is possible to isolate the various classes of organelles in a higher state of purity than that usually achieved from other tissues (15).

MATERIALS AND METHODS

Organelle Fractionation. Castor beans (*Ricinus communis* var. Hale) from McNair Seed Co., Plainview, Texas, were soaked for 24 hr in running tap water and germinated for 4.5 to 5 days in vermiculite at 30 C in the dark. For each preparation, 50 g of excised endosperm (from about 60 beans) were chopped in 60 ml ice-cold grinding medium (13% w/w sucrose, 150 mM Tricine, 1 mM EDTA [pH 7.5]) in an onion chopper for 10 min. After straining through one layer of nylon cloth and centrifuging at 270g for 10 min, the supernatant (about 60 ml) was distributed over three sucrose gradients (20 ml supernatant, 7 ml 15% sucrose, 3 ml linear 15-60%, and 3 ml 60%, all w/w sucrose containing 1 mM EDTA [pH 7.5]) which were centrifuged for 2 hr at 21,000 rpm in a Beckman SW 25.2 rotor. The gradients were fractionated from the top using an Isco model 640 while monitoring absorbance at 280 nm. The top 20 ml were collected in a measuring cylinder and the following 40 ml collected in 1.2-ml fractions. Assays for phosphorylcholine-glyceride transferase (21) revealed the peak fractions of ER, Cyt c oxidase (23) indicated mitochondria, and alkaline lipase (26) identified the glyoxysome peak. The two peak fractions of ER (about 28% w/w sucrose) from each gradient were pooled as were the two peak mitochondrial fractions (39% w/w sucrose) and two glyoxysomal fractions (about 51% w/w sucrose). To each of the three organelle samples were added 2 volumes 0.225 M KCl, 0.05 M Tricine (pH 7.5). After centrifugation at 40,000 rpm for 1 hr in a Beckman 65 rotor, the pellets were resuspended in 1 ml of 0.05 M Tricine (pH 7.5). This is essentially the procedure used by Huang (13) to obtain glyoxysomal membranes.

Lipid bodies (spherosomes) were obtained as a layer at the top of the 270g supernatant or the sucrose gradient. Proplastids (23, 28) were obtained by resuspending the 270g pellet in 5 ml 15% sucrose, 1 mM EDTA (pH 7.5), and fractionation on a sucrose gradient (5 ml 270g pellet, 50 ml linear 15-60% sucrose, 3 ml 60%, all w/w sucrose, containing 1 mM EDTA [pH 7.5]). The peak fractions (about 48% sucrose, triose phosphate isomerase, enzyme marker) were pooled, diluted, and pelleted as described above for the ER fractions.

Lipid Analysis. Lipid extracts of each organelle fraction were prepared by adding 10 ml of chloroform-methanol (2:1, v/v) to 0.5 ml of the suspension (7). After storage overnight at -20 C the extracts were washed twice with 0.2 volumes 0.1% (w/v) MgCl_2 and aliquots were taken for analysis.

Lipid classes were separated on thin layer plates obtained

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² Abbreviation: ER: endoplasmic reticulum.

from E. Merck (0.25 mm Silica Gel 60 on glass). The plates were prewashed by running overnight in chloroform-methanol (2:1, v/v) and activated for 30 min to 1 hr at 100 C. For application, each chloroform extract was taken to dryness under a stream of N₂ and redissolved in about 50 μ l chloroform-methanol (9:1, v/v). After application of this 50 μ l to the plate, the sample container was washed with an additional 50 μ l solvent which was also applied. For separation of phospholipids, the plate was developed in chloroform-methanol-acetic acid-water (65:50:5:3, v/v). Neutral lipids were separated in benzene-diethyl ether-ethanol-acetic acid (50:40:2:0.2, v/v).

Lipids were detected with I₂ vapor prior to gas chromatographic analysis. For phosphorus analysis, the lipids were detected with ninhydrin spray and molybdate spray (1). A series of standard lipids was run on the same plate to facilitate identification.

For phosphorus analysis, each region of the thin layer was scraped up and transferred to a small tube. Perchloric acid was added directly to the silica gel and phosphorus analyzed by a modification of the Bartlett method as described by Dittmer and Wells (7), except that the volume of each reagent was one-fourth of that stated.

The fatty acid composition of each lipid class was determined by gas chromatography of the methyl esters. The silica gel containing each lipid was scraped up and sucked into a Pasteur pipette containing a glass wool plug. The phospholipid was eluted from the silica gel with 2.5 ml methanol. To this was added 2.5 ml methanol containing 10% (v/v) H₂SO₄. Neutral lipids were eluted with chloroform, taken to dryness, and methylated in 5 ml 5% H₂SO₄ in methanol. Also, a known amount, 0.8 to 3.2 nmol, of nonadecanoic acid (C₁₉) or heptadecanoic acid (C₁₇) was added to each sample as an internal standard. Methanolysis was completed in the usual manner (1) and the methyl esters extracted with petroleum ether (30–60 C, b.p.). After drying down with N₂, the sample was redissolved in 5 to 100 μ l CS₂.

One μ l of each of the methyl ester samples was analyzed on a glass column (170 cm \times 2 mm) containing 15% DEGS on Chromasorb W-HP (100/200 mesh) at 180 C, 35 cm³/min He flow, in a Beckman GC 65 with flame ionization detector (250 C). The fatty esters were identified by comparison with a series of knowns (C_{8–24}, saturated and unsaturated) plotted with respect to the log of the retention time. Some neutral lipid samples containing ricinoleic acid were analyzed at a column temperature of 200 C. Methyl ricinoleate had a retention time of 50 min at 180 C and 19 min at 200 C (equivalent chain length = 29.2). Peak areas were determined by triangulation or by an Isco model 950 integrator and quantitated relative to the internal standard. Molar correction factors were obtained by using a calibration standard (Applied Sciences' FAME) and were similar to those reported by Allen (1).

Unesterified sterols from TLC were analyzed by gas chromatography. The glass column (1 m \times 2 mm) was packed with 10% SE-52 on Gas-chrom Q (Applied Science Labs, Pa.). At a temperature of 233 C and He flow of 55 cm³/min, the retention times relative to cholesterol were: cholestane, 0.51; stigmasterol, 1.42; and β -sitosterol, 1.64, which are comparable to relative retention times obtained on SE-30. Cholestane (about 1 μ g) was used as a quantitative internal standard.

All solvents were redistilled and glassware used for preparing methyl esters was washed with CHCl₃. Blank extracts were carried through the entire procedure and the resulting analysis accounted for some minor fatty acids. The blank values were subtracted from all analyses.

RESULTS

Phospholipid Composition of the Organelles. When lipid ex-

tracts of membrane pellets are subjected to TLC, phosphatidyl choline and phosphatidyl ethanolamine are the most prominent lipid classes visualized. The spot corresponding to cardiolipin is prominent only in mitochondrial extracts. Proplastids are the only organelles which yield visible phosphatidyl glycerol as well as yellow pigments in the solvent front. Traces of phosphatidyl serine may be detected with ninhydrin in all membrane extracts if the chromatograms are heavily loaded as well as very minor unidentified species in the vicinity of cardiolipin. Lysophosphatidyl choline and lysophosphatidyl ethanolamine were not seen using any detection methods (molybdate, ninhydrin, I₂), nor were glycolipids.

These visual observations are confirmed by phosphorus analysis of the lipid classes from each organelle fraction (Table I). Together phosphatidyl choline and phosphatidyl ethanolamine comprise more than 70% (on a molar basis) of the phospholipid in each organelle fraction. However, the relative amounts of the two phospholipids vary. The proportion of phosphatidyl choline is greatest in the glyoxysome membranes and least in the proplastids and mitochondria. Mitochondria from mammalian tissue (9) also have relatively less phosphatidyl choline than other organelles. Cardiolipin is a specific component of mitochondria from other organisms as it is in castor bean (Table I). This phospholipid, which contains 2 mol of phosphorus, has been shown to be located in the inner membranes of mitochondria from liver (5), and cauliflower (25). In their recent analysis of castor bean mitochondria, Ohmori and Yamada (27) reported that cardiolipin accounted for 5.4% of the membrane lipid. The major components were phosphatidyl choline and phosphatidyl ethanolamine.

Although phosphatidyl inositol is present in both the ER and the glyoxysome membrane, the amount in the latter is somewhat less. Phosphatidyl inositol is also the third most abundant phospholipid in microsomes and peroxisomes obtained from rat liver (9), as well as in nuclear membranes of onion (29), cauliflower outer mitochondrial membranes (25), and membranous vesicles from bean cotyledons (2).

Fatty Acid Composition of Phospholipids. The predominant fatty acids in each phospholipid class were linoleic (18:2), palmitic (16:0), oleic (18:1), stearic (18:0), and linolenic (18:3) acids. Other fatty acids such as laurate, myristate, palmitoleate, and long chain fatty acids, were detected. However, in any one phospholipid, such species accounted for no more than 4% of the total fatty acid and the standard deviations were usually greater than the mean percentages.

The fatty acid composition of the phosphatidyl choline obtained from the three major membranous fractions is represented in Figure 1. The composition of the glyoxysomal phosphatidyl choline is almost identical to that of the ER. In each,

Table I
Lipid Phosphorus

	Glyoxysome	Endoplasmic reticulum	Mitochondria	Proplastid
	mol % of lipid phosphorus in membrane ¹			
Phosphatidyl choline	51.4	45.3	33.1	35.6
Phosphatidyl ethanolamine	27.2	28.7	35.7	38.7
Phosphatidyl inositol	9.0	13.1	3.9	8.0
Phosphatidyl serine	1.5	2.3	2.2	4.6
Phosphatidyl ² glycerol	2.7	3.6	2.2	8.4
Cardiolipin	2.3	2.5	17.7	-
Origin	2.4	1.5	1.6	2.7
Front	1.5	1.0	1.7	1.7
Other	2.0	2.0	0.9	0.3
Number of experiments	4	3	4	2

¹ Recovery of phosphorus from thin layer chromatography was 87% or better

² Includes phosphatidic acid

the most abundant fatty acid is linoleate which comprises about 40% of the fatty acid on a molar basis. The fatty acid proportions in the mitochondrial phosphatidyl choline are similar to those in the other two membranes but with somewhat more linoleate. The predominant fatty acids in phosphatidyl choline are the same as those in other plant tissues (6). Where it has been examined, the saturated fatty acids have been found preferentially esterified at position 1 (6).

The fatty acid composition of phosphatidyl ethanolamine (Fig. 1) is slightly different from phosphatidyl choline in that there is less oleate in each case. However, its fatty acid content is similar in all three organelles.

Compared to the two major phospholipids of the organelles, phosphatidyl inositol contains more of the saturated fatty acid, palmitate (Fig. 1). There is no significant difference in the fatty acid composition of this phospholipid among the three organelle fractions. In other tissues, this lipid is also relatively rich in saturated fatty acid (2, 5).

The phosphorus analysis indicated that cardiolipin is confined to the mitochondria. This phospholipid, which bears four acyl

Table II
Lipid Composition Based On Fatty Acid Content

	Glyoxysome	Endoplasmic reticulum	Mitochondria
	mol %		
Phosphatidyl choline	33.4	23.9	24.5
Phosphatidyl ethanolamine	26.5	23.9	35.5
Neutral lipid	23.2	24.6	10.9
Phosphatidyl inositol	9.9	16.2	7.2
Phosphatidyl serine	1.2	3.3	3.2
Phosphatidyl glycerol	3.6	5.2	-
Cardiolipin	-	-	13.2
Origin	2.2	0.5	0.4

chains, is strikingly different from the other acyl lipids in its very high content of C₁₈ unsaturated acid (Fig. 1). Linoleate accounts for 75% of the fatty acid. Thus, cardiolipin of castor bean mitochondria is almost identical to that of liver (5).

Acyl Lipid Composition of the Organelles. A different perspective of the lipid composition is realized when the quantitation is based on fatty acid content (measured by gas chromatography) rather than phosphorus (Table II). Phosphatidyl choline is the most abundant acyl lipid in the glyoxysome membrane while phosphatidyl ethanolamine is more prominent in the mitochondria. The striking feature of this analysis is that 24% of the fatty acid is associated with the neutral lipid classes in the ER and glyoxysome membranes. This fraction, which runs between cardiolipin and the solvent front in the system used to resolve phospholipids, includes free fatty acid, monoglyceride, diglyceride, and triglyceride.

Fatty Acid Composition of Neutral Lipids. The extracts from each membrane fraction were chromatographed in a system which resolves the neutral lipids (Table III). Free fatty acid is a prominent feature in all membrane fractions. The mitochondria membranes contain the least amount of free fatty acid. 1,2-Diglyceride and triglyceride were detected in each of the three membranous organelles, especially in the ER. In contrast, triglycerides are the main constituents of the lipid bodies obtained from endosperm at the same stage of germination and ricinoleate is the dominant fatty acid component. These storage triglycerides separate into four classes in TLC as a result of differing content of hydroxylated fatty acid (22). Most of the triglyceride in dry seed is triricinolein although there is a significant amount (about 33%) containing one unhydroxylated fatty acid.

Very little ricinoleic acid was found in the membranous fractions either in the free form or as triglyceride. Compared to those in the dry seed, lipid bodies from germinating endosperm have significant amounts of free ricinoleate.

The free fatty acids present in each of the membrane fractions are the same as those found in the phospholipids (Fig. 2). However, the relative amounts are quite different. There is proportionally more stearate and oleate, and less linoleate. The free fatty acid of the lipid storage bodies is of another extreme, ricinoleate accounting for 32% of total lipid (Table III).

Palmitate and linoleate are the main fatty acids in the 1,2-diglycerides, as they are in the phospholipids.

The neutral acyl lipid content of membranes from other tissues is not assessed in most reports. Free fatty acids in amounts similar to those reported here have been found in nuclear membranes and ER (29). Free fatty acids detected in membranes from oat roots have been attributed to the action of phospholipases during tissue fractionation (17). This free fatty acid was accompanied by significant quantities of lysophosphatidyl choline and lysophosphatidyl ethanolamine which are not detected in castor bean membranes. Furthermore, the quantities of the individual fatty acids in these membranes are unlike those in the phospholipids (Fig. 2).

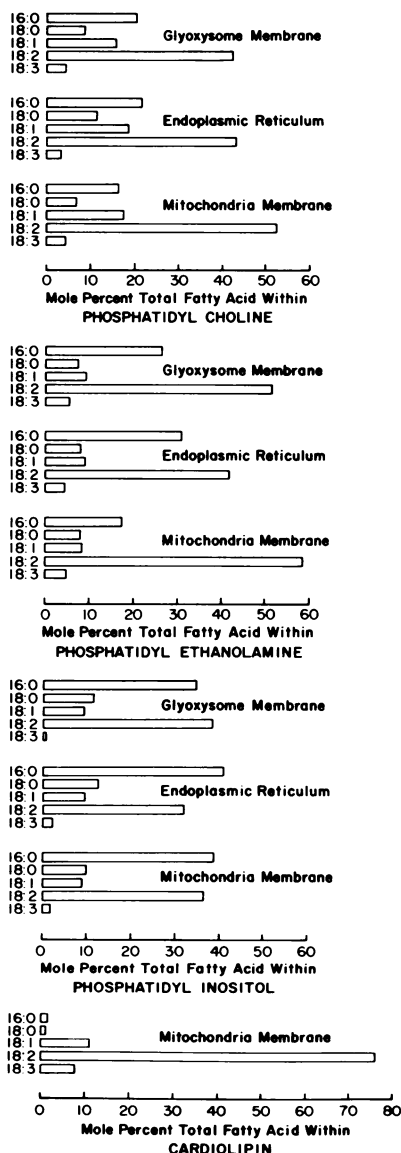


FIG. 1. Fatty acid composition of the individual phospholipids of organelle membranes. Palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2), linolenate (18:3). Average of four experiments.

Table III
Distribution of Fatty Acid Among Lipid Classes from Organelles

	Mitochondria membrane	Endoplasmic reticulum	Glyoxysome membrane	Five Day lipid bodies	Dry Seed extract
	mol % of total fatty acid				
Phospholipid	87.2	78.5	83.8	0.7	0.3
Free fatty acid					
Other than ricinoleate	9.6	13.5	12.6	5.4	trace
Ricinoleate	0.4	0.4	0.1	32.0	nd ¹
Diglyceride					
1,2	1.5	3.2	1.1	nd	nd
1,3	0.5	1.0	0.4	nd	nd
Triglyceride					
One or no ricinoleate	0.6	3.5	1.9	0.4	2.0
Two ricinoleate	nd	nd	nd	6.7	32.7
Three ricinoleate	0.1	nd	0.2	54.9	65.0

¹ not detected

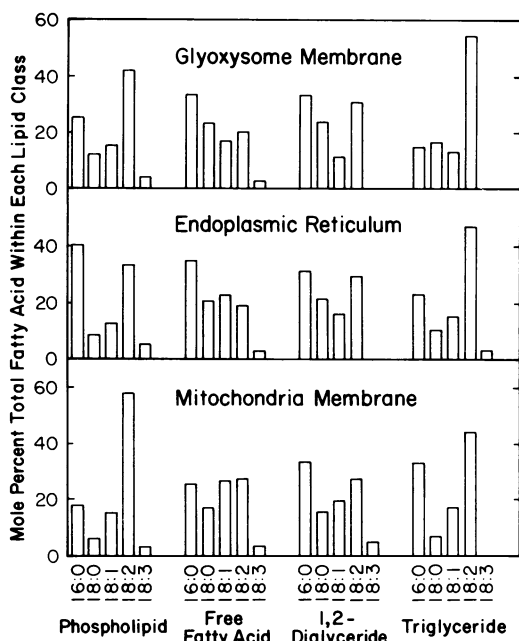


FIG. 2. Fatty acid compositions of neutral lipid classes from organelle membranes. Palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2), linolenate (18:3).

Sterols. A spot corresponding to sterol is obtained from each membrane fraction in the TLC of neutral lipids. When these spots were analyzed by gas chromatography, stigmasterol and β -sitosterol were found to be the only significant sterols, although compared to the amounts of acyl lipids, these sterols are minor constituents of the membranes (Table IV). Spots corresponding to steryl esters or glycosides were not observed. The endoplasmic reticulum contains the most sterol and the mitochondria the least, relative to protein, as shown, or also relative to phospholipids.

Comparable amounts of sterol are also found in microsomal pellets and mitochondria from etiolated bean leaves (4). β -Sitosterol and stigmasterol are the sterols of bean leaves whereas cholesterol is the only sterol in rat liver membranes.

DISCUSSION

The lipid compositions of castor bean mitochondria and ER are comparable to those of other plant and animal tissues. The glyoxysome represents a class of organelles (microbodies or peroxisomes) whose membrane composition has not previously been fully characterized. The results of this study show that this membrane is analogous to many other intracellular membranes (e.g. the outer mitochondrial, nuclear, and Golgi membranes) in

its similarity to the ER, at least with respect to lipids. The same three phosphoglycerides, those of choline, ethanolamine, and inositol, are the predominant components of both membranes. Each phospholipid has a characteristic fatty acid composition that is essentially constant in all membranes. This is yet another indication that there is a single site of origin of these phospholipids (15, 20, 21, 24) and that there is no selectivity of phosphoglycerides with respect to acyl content during the assembly of the glyoxysome membrane.

The acyl components of the membrane glycerides are probably synthesized *de novo* in the cytoplasm from acetate produced in the β oxidation of the ricinoleate (8), and further elaborated in the ER. The small amount of 1,2-diglyceride observed could represent an intermediate in this synthesis (20). The composition of each phospholipid would then be determined by the available diglyceride and by the selectivity of the respective synthetic enzymes (16).

Proplastids have a high ratio of phosphatidyl ethanolamine to phosphatidyl choline like the mitochondria, contain almost as much phosphatidyl inositol as the glyoxysomes, and contain more phosphatidyl glycerol than the other membranes. The latter is the most important phospholipid in chloroplasts (1). The proplastids develop complex internal structures in germinating castor bean but their function is unknown (29). They contain some of the enzymes normally involved in carbon fixation, such as ribulose diphosphate carboxylase and triose phosphate isomerase, but lack Chl (23, 28, 30).

Although the ER is the synthetic source of membrane lipids (3, 8, 15, 20, 21), no organelle membrane is completely identical to it. This includes the glyoxysome membrane in which the relative amounts of the phospholipids are altered. In addition, the glyoxysome membrane contains almost twice as much protein, even though the membranes are washed with 0.15 M KCl

Table IV
Summary Lipid Composition

	Endoplasmic reticulum	Glyoxysome membrane	Mitochondria membrane
	nmol/mg protein		
Phospholipid ¹	1243.1	653.8	547.4
Phosphatidyl choline ²	502.1	342.6	180.1
Phosphatidyl ethanolamine ²	405.3	193.5	188.3
Phosphatidyl inositol ²	206.4	56.9	17.0
Phosphatidyl glycerol ²	33.6	15.7	10.9
Cardiolipin ²	3.8	3.3	58.9
Free Fatty Acid ³	213.0	97.9	58.0
Diglyceride ³	34.3	6.1	6.3
Triglyceride ³	18.9	5.3	1.3
Stigmasterol ⁴	8.95	4.1	1.2
β -Sitosterol	6.3	2.4	1.0
Total acyl lipid	1631.15	828.45	614.7
Total lipid	1646.4	835.0	616.9

¹ based on phosphorus content

² from Table I

³ from Table III

⁴ G.C. analysis of free sterol from TLC

(Table IV). The glyoxysome membrane contains some proteins common to the endoplasmic reticulum, NADH Cyt *c* reductase (9), and Cyt *b*₅ (Donaldson, unpublished), but it also bears at least one constitutive protein, the lipase (26). Several enzymes of the glyoxylate cycle are associated with the membrane, but in contrast to the lipase, they can be removed by 0.15 M KCl (13).

Several lines of evidence indicate that glyoxysomes are derived from the ER. The similarities in lipid composition, the site of phospholipid synthesis, and the presence of common proteins all suggest that the membrane is produced by the ER. Also, Gonzalez (10) has shown that early in germination during the development of glyoxysomes, a large fraction of the activity of the glyoxysomal enzymes, citrate synthetase, and malate synthetase, is present in the ER. The observations of direct connections between the ER and glyoxysomes give a morphological basis for the biochemical findings (30).

Although the evidence suggests that during germination, glyoxysomes are assembled while in direct continuity with the ER (3), the data do not preclude some exchange of material and perhaps differentiation independent of the endoplasmic reticulum. This would involve the passage of organelle components through the cytoplasm, a possibility that has been suggested for the biogenesis of rat liver peroxisomes to explain the appearance of newly synthesized apocatalase in the supernatant fraction prior to incorporation in the organelle (18). To explain the quantitative differences in lipid and protein content of the glyoxysome membrane, one might imagine that only specific regions of the ER are diverted for glyoxysome biogenesis.

On the other hand, one could invoke selective exchange of phospholipids mediated by specific phospholipid carrier proteins which are known in plants (14). A corollary of this idea is that glyoxysomal biogenesis may not be a concerted process. There is evidence (from rat liver) that the smooth ER is responsible for lipid synthesis (12) while the membrane proteins are synthesized in the rough (11). The proteins such as glucose-6-phosphatase are then inserted into the pre-existing lipid membrane (19). These observations suggest mechanisms whereby the glyoxysomal membrane could become differentiated while detached from the ER. The possibility remains that proteins could pass through the membrane of pre-existing organelles, leading to changes in the content of soluble enzymes as well.

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