

Acquisition of Membrane Lipids by Differentiating Glyoxysomes: Role of Lipid Bodies

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Abstract. Glyoxysomes in cotyledons of cotton (*Gossypium hirsutum*, L.) seedlings enlarge dramatically within 48 h after seed imbibition (Kunce, C. M., R. N. Trelease, and D. C. Doman. 1984. *Planta (Berl.)*. 161:156–164) to effect mobilization of stored cottonseed oil. We discovered that the membranes of enlarging glyoxysomes at all stages examined contained a large percentage (36–62% by weight) of nonpolar lipid, nearly all of which were triacylglycerols (TAGs) and TAG metabolites. Free fatty acids comprised the largest percentage of these nonpolar lipids. Six uncommon (and as yet unidentified) fatty acids constituted the majority (51%) of both the free fatty acids and the fatty acids in TAGs of glyoxysome membranes; the same six uncommon fatty acids were <7% of the acyl constituents in TAGs extracted from cottonseed storage lipid bodies. TAGs of lipid bodies primarily were composed of palmitic, oleic, and linoleic acids (together 70%). Together, these three major storage fatty acids were <10% of both the free fatty acids and fatty acids in TAGs of glyoxysome membranes.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) constituted a major portion of glyoxysome membrane phospholipids (together 61% by weight). Pulse-chase radiolabeling experiments *in vivo* clearly demonstrated that ¹⁴C-PC and ¹⁴C-PE were synthesized from ¹⁴C-choline and ¹⁴C-ethanolamine, respectively, in ER of cotyledons, and then transported to mitochondria; however, these lipids were not transported to enlarging glyoxysomes. The lack of ER involvement in

glyoxysome membrane phospholipid synthesis, and the similarities in lipid compositions between lipid bodies and membranes of glyoxysomes, led us to formulate and test a new hypothesis whereby lipid bodies serve as the dynamic source of nonpolar lipids and phospholipids for membrane expansion of enlarging glyoxysomes. In a cell-free system, ³H-triolein (TO) and ³H-PC were indeed transferred from lipid bodies to glyoxysomes. ³H-PC, but not ³H-TO, also was transferred to mitochondria *in vitro*. The amount of lipid transferred increased linearly with respect to time and amount of acceptor organelle protein, and transfer occurred only when lipid body membrane proteins were associated with the donor lipid bodies. ³H-TO was transferred to and incorporated into glyoxysome membranes, and then hydrolyzed to free fatty acids. ³H-PC was transferred to and incorporated into glyoxysome and mitochondria membranes without subsequent hydrolysis.

Our data are inconsistent with the hypothesis that ER contributes membrane lipids to glyoxysomes during postgerminative seedling growth. Instead, the data support a novel source for glyoxysome (peroxisome) membrane lipids; lipid bodies, which house storage lipids that are converted to carbohydrate during heterotrophic seedling growth, also provide enlarging glyoxysomes with nonpolar lipids and phospholipids to accommodate membrane expansion. A working model depicting the origin and intracellular trafficking of membrane lipids for enlarging cottonseed glyoxysomes is presented.

GLYOXYSOMES of oilseeds are specialized peroxisomes which, through the cooperative action of enzymes catalyzing fatty acid β -oxidation and the glyoxylate cycle, play a pivotal role in the conversion of storage oil into carbohydrate (gluconeogenesis) for seedling growth (2, 3, 25, 29). Glyoxysomes in cotyledons of cotton seedlings en-

large in volume sevenfold within 48 h after seed imbibition (see Fig. 1; see also reference 30). Similar enlargement events have been observed ultrastructurally in cotyledons of other oilseeds (25, 39, 42, 44, 53). The increase in glyoxysome volume is presumably to accommodate the rapid accumulation of matrix proteins. Many glyoxysomal enzymes are known to increase dramatically in activities (2, 6, 18, 25, 29, 44, 50), absolute amounts of protein (18, 37, 50), and levels of transcripts (1, 14, 18, 37, 43, 50, 54) concomitant with

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glyoxysome enlargement. It follows that a significant amount of membrane lipid must also become available to glyoxysomes for membrane expansion.

While much attention has been devoted toward understanding the biogenesis of peroxisomal matrix components, comparatively little effort has been directed toward investigating the assembly of peroxisome membranes, particularly with respect to membrane lipids. Membrane lipids are primarily synthesized in the ER in animal cells (4, 16; see references therein), plant cells (30; see references therein), and yeasts (8; see references therein). Peroxisomes apparently lack the ability to synthesize their own membrane lipid (4, 8, 10, 16, 31–33). All models of peroxisome biogenesis that consider the origin of peroxisome membrane lipids postulate the transfer of newly synthesized phospholipid to peroxisomes from ER (21, 25, 31, 32, 46). However, there are almost no data in any of these systems to support this part of the biogenetic models. One exception was a report that radiolabeled phosphatidylcholine (PC)¹ was transported from ER to glyoxysomes in endosperm tissue of castor beans (28). Endosperm glyoxysomes do not enlarge during reserve oil mobilization; instead, they are believed to proliferate in number by vesiculation from segments of ER after seed germination (21, 32). Therefore, results obtained for endosperm tissue on the biogenesis of glyoxysome membranes are not likely to be applicable to other peroxisomal systems such as those in liver (31), yeasts (52), and seedling cotyledons (30, 46, 54) where biogenesis is via an elaboration of preexisting organelles.

Enlarging cottonseed glyoxysomes were shown to be incapable of synthesizing their own PC and phosphatidylethanolamine (PE) (10); i.e., they did not possess choline- or ethanolaminophosphotransferase activities, nor were they able to convert exogenously supplied radiolabeled choline or ethanolamine into radiolabeled PC or PE in vivo. As in mammalian, yeast, and endosperm systems, ER in cotyledons of cotton seedlings was confirmed to be the primary intracellular site of PC (and PE) synthesis (10).

To identify the cellular origin of membrane lipids for enlarging cottonseed glyoxysomes, we integrated results from several experimental approaches: first, nonpolar and polar lipid compositions of carbonate-washed membranes from highly purified glyoxysomes were determined and compared to the lipid compositions of other organelle fractions; second, pulse-chase experiments with radiolabeled choline and ethanolamine were done to trace the synthesis and intracellular trafficking of PC and PE in vivo; and third, an in vitro lipid transfer system was developed to test for direct transfer of radiolabeled nonpolar and polar lipids from lipid bodies to membranes of highly purified glyoxysomes. Inconsistent with current views on the origin of peroxisome membrane lipids, our data support an entirely different means by which glyoxysomes (peroxisomes) acquire membrane lipids; i.e., lipid bodies, besides providing the growing seedling with a carbon and energy source, supply glyoxysomes with phospholipids and certain nonpolar lipids that are preferentially apportioned into expanding glyoxysome membranes.

1. *Abbreviations used in this paper:* FFA, free fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; TAG, triacylglycerols; TO, triolein.

Materials and Methods

Chemicals

Potassium phosphate, potassium chloride, magnesium chloride, EDTA, EGTA, PMSF, calcium chloride, sodium sulfate, hexane, diethyl ether, tetrahydrofuran, formic acid (88%), glacial acetic acid, ammonium hydroxide, chloroform, methanol, and sucrose (ribonuclease free) were from J. T. Baker Chemical Co. (Phillipsburg, NJ). NADH, antimycin A, cytochrome c, Tricine, MES, choline chloride, ethanolamine hydrochloride, PC, PE, lysoPE (palmitoyl), α -phosphatidyl (*N*-palmitoyl) ethanolamine (dipalmitoyl), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidate, phosphatidylserine, nonpolar lipid standards (cholesterol, cholesterol oleate, triolein (TO), tripalmitin, oleate, monopalmitin, dipalmitin (sn-1,2 and sn-1,3), and boron trifluoride methanol) were from Sigma Chemical Co. (St. Louis, MO.) [methyl-¹⁴C] Choline chloride (58.5 mCi/mmol), [2-palmitoyl-9,10-³H(*N*)] PC, L- α -dipalmitoyl (50 Ci/mmol), and [9,10-³H(*N*)] TO (26.8 Ci/mmol) were from duPont New England Nuclear Research Products (Boston, MA). [1,2 ethanolamine-¹⁴C] Ethanolamine hydrochloride (100 mCi/mmol) was from ICN Biomedicals, Inc. (Costa Mesa, CA). LR white (hard grade) was from Ernest F. Fullam, Inc. (Latham, NY). Osmium tetroxide (4%) and glutaraldehyde (25%) were from Electron Microscopy Sciences (Fort Washington, PA).

Plant Material

Cotton seeds, *Gossypium hirsutum*, L. cv Coker 100A glandless (kindly provided by Dr. Donald Hendrix, USDA Western Cotton Research Laboratory, Phoenix, AZ), were soaked in distilled water with aeration 4 h (30°C). Imbibed seeds (if not homogenized and fractionated for analyses) were placed on moistened filter paper (No. 1, Whatman Inc., Clifton, NJ), rolled into "scrolls" which were placed vertically in distilled water for germination and growth in the dark at 30°C. For radiolabeling experiments, imbibed seeds were first decanted, then placed on moistened filter paper in 15-cm glass petri plates for germination and growth in the dark at 30°C.

Preparation of Glyoxysomes

All concentrations given are final concentrations unless indicated otherwise. All percent sucrose concentrations are percent wt/wt. Cotyledons of 4, 24, or 48-h-old cotton seedlings were chopped into ~1-mm pieces in a plastic dish on ice with a modified electric knife (fitted with two stainless steel single-edge razor blades) in ice-cold medium (1 vol/g fresh weight) containing 100 mM potassium phosphate (pH 7.2), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and 400 mM sucrose. For analysis of membrane lipids, cotyledons of 250 seeds or seedlings (~30–40 g fresh weight depending on age) were required to yield sufficient quantities of glyoxysome membranes. For other experiments, cotyledons from 130 seedlings were adequate. The homogenate was filtered through one layer of Miracloth (pre-soaked with homogenizing medium) and centrifuged 10 min at 640 g (4°C) (model JS-13 rotor, Beckman Instruments, Inc., Fullerton, CA). The clarified homogenate was centrifuged in the same rotor for 20 min at 4,000 g (4°C). Pellets, enriched with glyoxysomes and mitochondria (10), were resuspended in fresh homogenizing medium (1 ml medium to 4 g initial fresh weight of cotyledons). Resuspended pellets (2.5 ml per gradient) were layered onto 100 mM potassium phosphate-buffered (pH 7.2) linear sucrose gradients (33 ml of 20–59% underlaid with 5 ml 59% sucrose) and centrifuged 45 min at 50,000 g (4°C) (model VTi 50 rotor, Beckman Instruments, Inc.) using the acceleration/deceleration program previously described (11). 1.2-ml fractions were collected using a density gradient fraction collector (model 640, ISCO, Lincoln, NE) and analyzed for enzyme activity and protein content. Glyoxysomes and mitochondria were collected from the same gradients.

Alternatively, highly purified glyoxysomes were isolated in linear metrizamide gradients (20–55%) and fractionated dropwise from the bottom exactly as described for cottonseed cotyledons by Turley and Trelease (49), except cotyledons were soaked in ultrapure water (Nanopure II, Barnstead Co., Boston, MA) for 30 min before homogenization in 100 mM potassium phosphate (pH 7.2), 400 mM sucrose.

Preparation of Organelle Membranes

Organelle membranes were stripped free of matrix material according to the method of Fujiki et al. (20), with slight modifications (19). Peak mito-

chondria- or glyoxysome-containing fractions (2–3) were pooled, diluted with 2 vol of ice-cold 100 mM potassium phosphate (pH 7.2), vortexed vigorously for 60 s, and incubated on ice for 30 min. Membranes were sedimented (100,000 g, 45 min, 4°C, model 70.1 Ti rotor, Beckman Instruments, Inc.), washed by resuspension in 1 ml, then diluted to 7 ml with ice-cold 100 mM Na₂CO₃ (pH 11.5). Samples were vortexed, incubated on ice for 30 min, and centrifuged as above. Membrane pellets were resuspended in 0.8 ml ice-cold homogenizing medium or processed for transmission electron microscopy (TEM) as described below.

Lipids were extracted from carbonate-washed membranes by addition of chloroform/methanol (1:2, vol/vol) to yield a final ratio of chloroform/methanol/water (1:2:0.8) (reference 5). After 30 min, the mixture was separated into two phases by adding 1 ml chloroform and 2 ml of 1 M KCl followed by centrifugation (5 min, setting 7, GT2 Tabletop centrifuge, IEC, Needham Heights, MA). The lower chloroform phase was washed two additional times with 2 ml 1 M KCl, bubbled with N₂, and stored at –20°C.

Preparation of Lipid Bodies

Lipid bodies were purified by the flotation method of Qu et al. (41). Cotyledons of 24-h-old cotton seedlings (50, ~6 g fresh weight) were homogenized in 10 ml 100 mM Tricine HCl, 10 mM KCl, 1 mM EDTA, 600 mM sucrose (pH 7.5) in the same manner as for glyoxysomes. The filtered homogenate was centrifuged at 10,000 g (model JS-13 rotor; Beckman Instruments, Inc.) for 30 min (4°C) and the fat pad was collected with a stainless steel spatula and suspended in 3 ml homogenizing medium. 10 ml of flotation medium (same as homogenizing medium, except with 500 instead of 600 mM sucrose) was layered over the suspended lipid bodies and the sample was centrifuged as above. The fat pad was collected and washed once more in the same manner. The resulting fat pad was collected and the volume adjusted to 0.8 ml with homogenizing medium. Lipids were extracted as described above for membranes and stored (–20°C, under N₂) for later analyses.

For preparation of radiolabeled lipid bodies, the lipid bodies collected after the third centrifugation were suspended in 2 ml homogenizing medium and split into two 15-ml corex tubes. ³H-TO (10 μCi) or ³H-PC (10 μCi) were added to the lipid bodies which were sonicated 10 s (model 1200 sonicator bath, 50/60 MHz, Branson Ultrasonics Corp., Danbury, CT), vortexed briefly, and placed on ice for 30 min. 10 ml of flotation medium were layered over the lipid bodies which were then centrifuged at 10,000 g (30 min, 4°C). The fat pads were collected, suspended in 1 ml of 100 mM potassium phosphate, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 400 mM sucrose (pH 7.2), and used as the source of radiolabeled lipid bodies for in vitro lipid transfer experiments. 20% of the added ³H-TO and 10% of the added ³H-PC was routinely recovered in the floated lipid bodies.

Radiolabeled synthetic lipid bodies were prepared as follows. Lipids were extracted from lipid bodies (floated three times) as described above (see *Preparation of Organelle Membranes*), evaporated to dryness under N₂, and suspended in 5 ml diethyl ether. Lipids in ether were sonicated (model 1200 sonicator bath, 50/60 MHz, Branson Ultrasonics Corp.) under a stream of N₂. Radiolabeled TO (2 μCi) or PC (1 μCi) and 1 ml of potassium phosphate homogenizing medium were added to the lipids (in ether). As the mixture was sonicated, the ether evaporated. After ~3 min the mixture became noticeably turbid, indicating the formation of synthetic lipid bodies (triacylglycerol [TAG] droplets surrounded by a monolayer of phospholipid but without native lipid body proteins in aqueous solution).

Analysis of Cellular Fractions

Antimycin A-insensitive cytochrome c reductase was assayed at 550 nm using an extinction coefficient of 21 mmol⁻¹ cm⁻¹ as previously described (9). Catalase activity was assayed as described by Ni et al. (38) in a final volume of 1 ml. Isocitrate lyase (6) and malate synthase (48) activities were assayed as described. Cytochrome c oxidase was assayed as described by Tolbert et al. (45), including a 1-min preincubation at 30°C with 1.0% (wt/vol) digitonin. Protein content was determined by the Coomassie blue dye-binding method (Bio-Rad Laboratories, Richmond, CA) using bovine plasma γ-globulin as a standard. Assays were performed using a spectrophotometer (model DU-64, Beckman Instruments Inc.). Sucrose concentrations were measured using a refractometer (model Abbe 2C, Bausch & Lomb Inc., Rochester, NY).

Microscopy

Catalase cytochemistry was performed on fixed tissue segments (~1 mm³)

of cotyledons from imbibed seeds or 48-h-old seedlings as previously described (30), except the tissue was dehydrated in a graded ethanol series and infiltrated with LR White (hard grade) (London Resin Co. Ltd., Woking, Surrey, England) at 4°C. Polymerization was at 57°C (overnight) in a corked glass vial under N₂. Tissue segments were cut out and mounted onto acrylic rods for sectioning.

Glyoxysomes were prepared for microscopic examination based on methods previously described (11), with some modifications. The fraction containing the highest catalase-, isocitrate lyase-, and malate synthase-specific activities was fixed (4°C) by diluting with 2 vol of 100 mM potassium phosphate (pH 7.2), 2.5% (vol/vol) glutaraldehyde (stepwise addition of 0.5-ml aliquots over a 30-min period) and incubating for 30 min more on ice. The fixed organelles were collected by centrifugation (13,000 g, 30 min, 4°C, model JS-13 rotor, Beckman Instruments, Inc.) onto a membrane filter (GA-6 Metrical, 13-mm disc, 0.45-μm pore size [Gelman Sciences Inc., Ann Arbor, MI], placed at the bottom of a 15-ml corex tube) and covered with a drop of warm 5% (wt/vol) noble agar. The sample was washed three times (15 min each) with 100 mM potassium phosphate (pH 7.2) and postfixed in 100 mM potassium phosphate, 2% (vol/vol) osmium tetroxide for 45 min at room temperature. The organelles were dehydrated in a graded ethanol series at room temperature, infiltrated with and embedded in LR white (hard grade) at 4°C. After polymerization (57°C, overnight), pieces of the filter were cut out and mounted onto acrylic rods so that the plane of section was perpendicular to the layer of organelles.

Carbonate-washed glyoxysome membranes were fixed as above after encasing the pellet in a drop of 5% noble agar. The pellet was then processed for TEM as described above for glyoxysomes.

Lipid bodies (washed three times by flotation) were fixed as above after immobilizing a piece of the fat pad (~1 mm²) in 5% noble agar. The lipid bodies were then processed for TEM as described above for glyoxysomes.

Semithin sections (500 nm) or thin sections (60–90 nm) were cut with a glass knife using an ultramicrotome (model MT-6000 XL, RMC, Inc., Tuscon, AZ). Semithin sections were dried onto a glass slide and viewed directly with a phase-contrast microscope (Carl Zeiss, Inc., equipped with a 63× oil-immersion planapochromat lens (NA 1.4), or stained 3 min with 1% methylene blue and examined with a compound light microscope (model BH-2, Olympus Corporation of America., La Palma, CA) fitted with a 60× oil-immersion lens.

Thin sections were poststained with 2% aqueous uranyl acetate (3 min) followed by 2.5% lead citrate (1 min), and examined at 60 kV in a transmission electron microscope (model EM201, Philips Electronic Instrs. Co., Mahwah, NJ).

Lipid Analyses

The Iatroscan TLC/flame ionization detection (FID) (Iatroscan TH-10) system was used as previously described (23) for quantification of nonpolar lipids in membranes and organelle fractions. Separations were carried out on fused silica rods (Chromarods-SII, stored in 6 M HNO₃). Lipid samples in chloroform (1 μl) were spotted onto rods (previously washed five times in glass-distilled water and dried 1 h at 120°C), then equilibrated at 52% relative humidity (by 15-min incubation over saturated Na₂Cr₂O₇ solution) before development in the first solvent system, hexane/diethyl ether/formate (80:20:2, vol/vol/vol) for 35 min. Hexane was redistilled for all lipid analyses. The rods, except for the origin containing polar lipids, were scanned and positions of the individual nonpolar lipid classes were detected (flame ionization) and recorded. For separation of phospholipids, the same rods were equilibrated for 15 min at 84% relative humidity (over a saturated KBr solution) before development in the second solvent system, chloroform/methanol/water (80:35:3, vol/vol/vol) for 50 min, after which the entire rods (including the origin) were scanned and positions of the phospholipid classes were detected and recorded. Identification of lipids was made by comparing mobilities of standards; quantification of lipids was done by integrating peak areas and computing values from standard curves (a standard curve was generated for each nonpolar and polar lipid class).

The peaks corresponding to PG, PI, and PE overlapped somewhat in the solvent system used for Iatroscan TLC/FID. Therefore, two-dimensional TLC was used to obtain accurate proportions of phospholipid classes in organelle fractions. Nonpolar lipids were first separated from polar lipids by spotting samples (in ~40 μl chloroform) onto silica gel G plates (0.25-mm silica gel G, 10 × 20 cm Uniplates; Analtech, Inc., Newark, DE) and developing in hexane/diethyl ether/formate (80:20:2, vol/vol/vol) for 30 min. The origin (containing the phospholipids) was scraped into a glass vial containing 5 ml chloroform/methanol (2:1, vol/vol) vortexed vigorously, and filtered (No. 54 filter paper, Whatman Inc.). The vial and the filter were

washed four times with 3 ml chloroform/methanol (2:1, vol/vol). The solvent was evaporated with a stream of N₂ and the residue was suspended in 50 μ l chloroform/methanol (2:1, vol/vol). The phospholipids were spotted onto 20 \times 20 cm silica gel G plates (Analtech, Inc.) and developed in the first solvent system (CHCl₃/methanol/7 M NH₄OH, 65:30:4, vol/vol/vol) for 60 min, rotated 90°, and developed in the second solvent system (CHCl₃/methanol/acetic acid (glacial)/water (ultrapure), 85:12.5:12.5:3, vol/vol/vol/vol) for 90 min (13). Phospholipids were visualized by charring (13) and identified by their mobility as compared with commercial standards. Phospholipids were quantified by reflectance scanning densitometry (Visage 60 video scanning densitometer, Millipore Corp., Bedford, MA). A standard curve for each phospholipid class was generated and amounts of phospholipids in lipid bodies, mitochondrial membranes, and glyoxysome membranes were estimated by computing values (integrated intensity, integrated area of the spot \times intensity index) from standard curves.

Gas chromatographic separations of fatty acid methyl esters were performed to analyze the acyl composition of TAG in lipid bodies and in carbonate-washed glyoxysome membranes, and the free fatty acids (FFA) in carbonate-washed glyoxysome membranes. Fatty acid methyl esters were prepared as follows. Nonpolar lipids were separated by development in hexane/diethyl ether/formate (80:20:2, vol/vol/vol) on silica gel G TLC plates and spots corresponding to the lipid body TAG and glyoxysome membrane TAG and FFA were scraped and transferred to glass vials containing 5 ml hexane. The samples were vortexed vigorously and filtered (No. 54 filter paper, Whatman Inc.). The vial and the filter were washed four times with 3 ml hexane. The solvent was evaporated with a stream of N₂ and the residue, after 10 min under in-house vacuum, was suspended in 2 ml boron trifluoride methanol. 1 ml of tetrahydrofuran was added to TAG samples to effect solubilization. TAG samples were refluxed 2 h and FFA samples were refluxed for 30 min at 70°C (13). After cooling, 2 ml hexane was added to the samples, which were vortexed lightly and centrifuged (5 min, setting 7, model GT2 Tabletop centrifuge, IEC, Inc.). The hexane phase was collected, washed once with 2 ml 5% (wt/vol) NaCl, once with 2 ml 2% (wt/vol) KHCO₃ and dried by passing over sodium sulfate columns (packed in pasteur pipettes, stored at 120°C until used). Columns were washed with 3 ml hexane. The solvent was evaporated with a stream of N₂ and fatty acid methyl esters were resuspended in 100 μ l hexane and stored under N₂ at -20°C until analysis. Methyl esters were separated by gas chromatography (model 5840A, Hewlett-Packard Co., Palo Alto, CA) on a 30-M fused silica column (Supelcowax-10, Supelco, Inc., Bellefonte, PA) using N₂ as the carrier gas at an oven temperature of 200°C. Fatty acids were identified by reference to commercial standards.

To identify the lipids in extracts of cellular fractions from *in vivo* radiolabeling experiments or from *in vitro* lipid transfer experiments, a combination of one-dimensional TLC and autoradiography was used. Samples in \sim 40 μ l chloroform were applied to silica gel G plates. Nonpolar lipids were separated by development in hexane/diethyl ether/formate (80:20:2, vol/vol/vol). For separation of individual phospholipids, these plates were developed in the same direction in chloroform/methanol/water (80:35:3, vol/vol/vol). Lipids were visualized by exposure to iodine vapor for 1 min and by autoradiography. For detection of lipids containing ³H, plates were sprayed with EN³HANCE (New England Nuclear, Boston, MA) before exposure of film (XAR 5; Eastman Kodak Co., Rochester, NY). Radioactivity was quantified by scraping spots into 15-ml scintillation vials and suspending in 10 ml of cocktail (4a20, Beckman Instruments, Inc.); silica gel particles were allowed to settle overnight and radioactivity was determined by liquid scintillation counting (model LS 8000 liquid scintillation counter, Beckman Instruments, Inc.). Identification of radioactive phospholipids was made by comparing mobilities of radioactive lipid spots to those of standards.

Radiolabeling In Vivo

Incorporation of (methyl-¹⁴C) choline chloride and (1,2 ethanolamine-¹⁴C) ethanolamine hydrochloride into ¹⁴C-PC and ¹⁴C-PE was performed as follows. Soaked (4 h) seeds were deoiled and grown on moist filter paper in petri plates for 18 h (radicles were 0.5–1.0 cm long). 30 seedlings were washed three times in ultrapure water, blotted dry, and then placed on filter paper in 150 \times 25 mm plastic petri plates containing 8 ml ultrapure water. Each seedling was radiolabeled by dispensing undiluted radioisotope (0.2 μ Ci, in 2 μ l) onto the inner surface of the partially unfolded cotyledons. Seedlings were incubated for 30 min with ¹⁴C-ethanolamine or 1 h with ¹⁴C-choline and homogenized in potassium phosphate-buffered media as described above (see *Preparation of Glyoxysomes*). Clarified homogenates (640-g, 10-min supernatants) prepared from 60 radiolabeled cotyledons

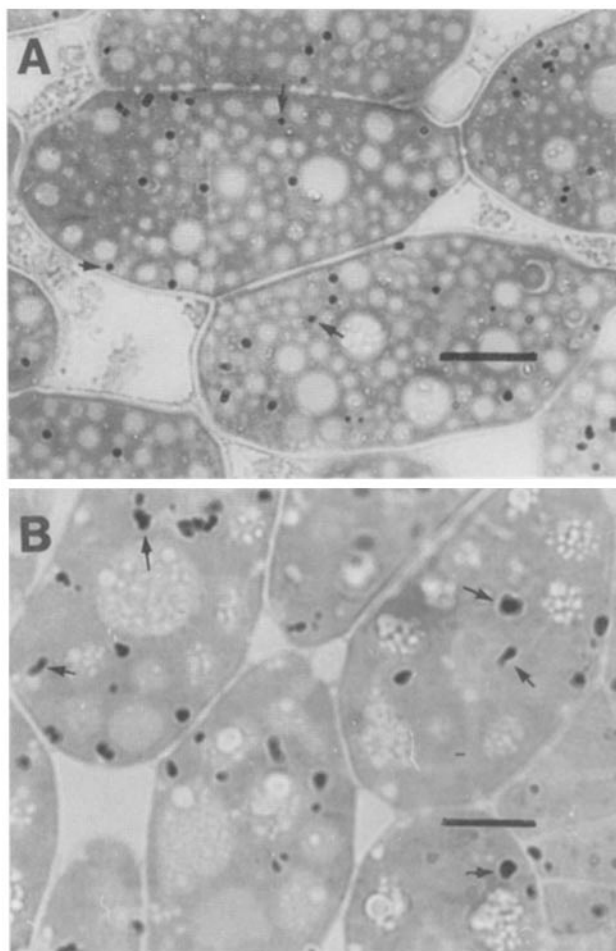


Figure 1. Phase-contrast micrographs of plastic-embedded sections of cotyledons from imbibed cotton seeds (A) or 48-h-old seedlings (B). Cotyledon segments were prepared to cytochemically localize catalase activity (with DAB). Catalase-containing glyoxysomes (arrows) are obviously enlarged in B as compared with those in A. Bars, 10 μ m.

were subjected to sucrose density gradient centrifugation as described above. After centrifugation and fractionation (into 2-ml fractions), lipids were extracted from gradient fractions (1-ml aliquots) by adding 5 ml chloroform/methanol/water (1:2:0.3, vol/vol/vol). The chloroform phase containing radioactive lipid products was washed twice with 2 ml 1 M KCl. The remaining chloroform phase was transferred to a 7-ml glass scintillation vial and evaporated to dryness with a stream of N₂. The residue was suspended in 3 ml scintillation cocktail and radioactivity measured by liquid scintillation counting, or alternatively samples were dissolved in a small volume of chloroform for analysis by TLC.

To follow the intracellular transport of radiolabeled PE or PC, pulse-labeled cotyledons were incubated 2, 12, or 24 h with 1 M nonradioactive ethanolamine or 1.7 M nonradioactive choline (both in water). Clarified homogenates (640-g, 10-min supernatants) prepared from 60 cotyledons were subjected to sucrose density gradient centrifugation, lipid extraction, and analyses as described above.

Membrane Lipid Transfer In Vitro

Organelle fractions were prepared fresh and used immediately for lipid transfer experiments (glyoxysomes and mitochondria were prepared from cotyledons of 48-h-old seedlings, whereas lipid bodies were prepared from cotyledons of 24-h-old seedlings). Glyoxysome fractions (peak catalase activity, 2–3 tubes/gradient) or mitochondria fractions (peak cytochrome c oxidase activity, two tubes) from two gradients were pooled and dialyzed (Spectra/por dialysis tubing, 23 mm, 6,000–8,000 MWCO, Spectrum Medical Industries, Inc., Los Angeles, CA) 2 h against 1 liter of 100 mM potas-

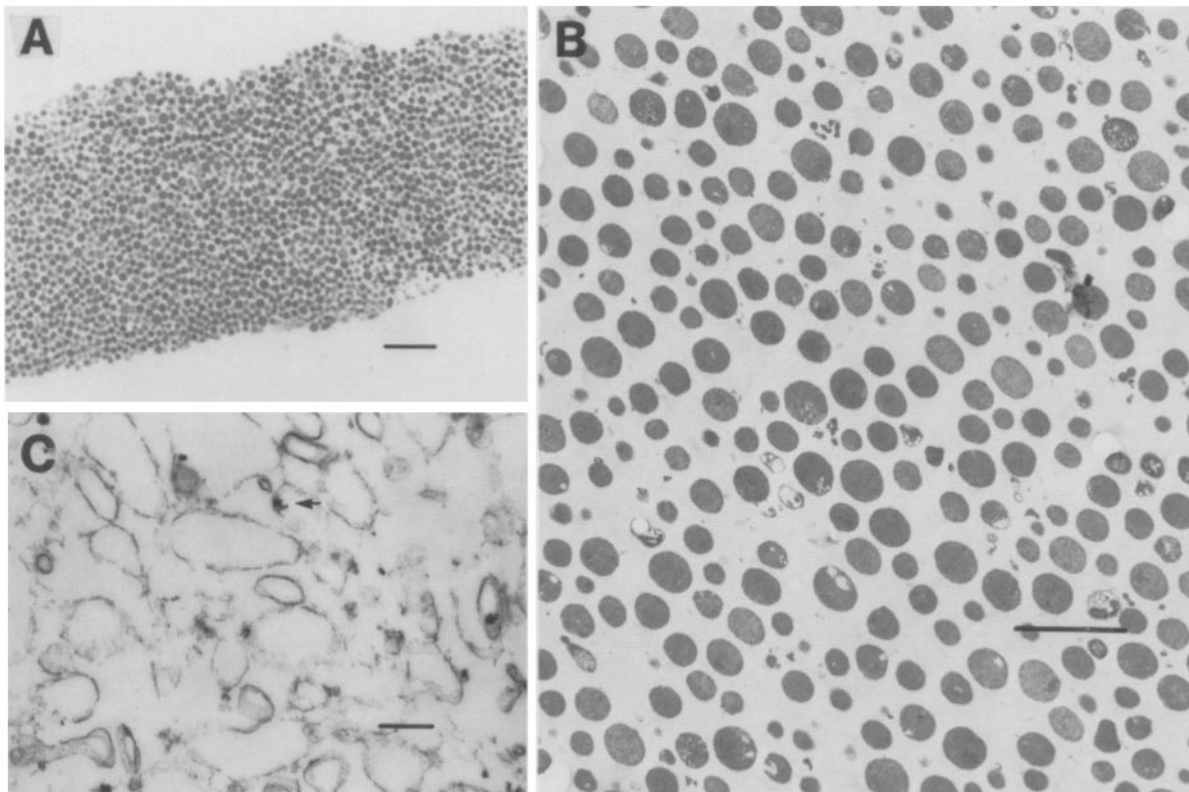
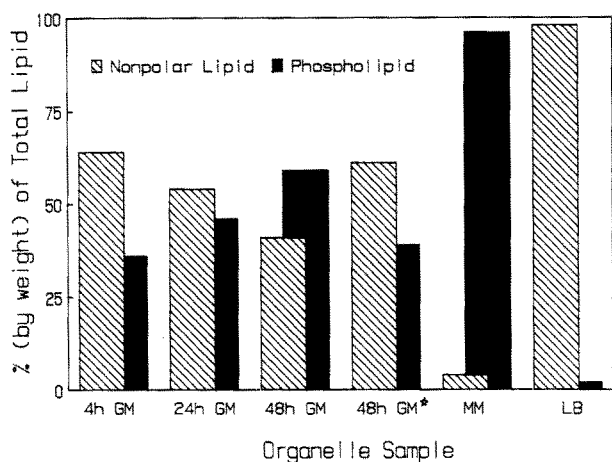


Figure 2. Light (A) and electron (B) micrographs of the glyoxysome fraction collected on a membrane filter, and membranes (C) prepared by diluting these glyoxysomes in 100 mM potassium phosphate (pH 7.2) followed by washing in 100 mM Na₂CO₃ (pH 11.5). Membranes were generally stripped free of matrix material although some aggregate material (tentatively identified as "cores," arrows) was visible. Bars: (A) 15 μ m; (B) 3 μ m; (C) 0.5 μ m.

sium phosphate (pH 7.2), 100 mM sucrose at 4°C. Organelles were concentrated by centrifugation at 10,000 g for 20 min (model JS-13 rotor, Beckman Instruments, Inc.) and resuspended in potassium phosphate-containing homogenizing medium as described above (see *Preparation of Glyoxy-*



* diluted in 100 mM Na carbonate only

Figure 3. Comparisons of the proportion of nonpolar lipid in: (a) carbonate-washed glyoxysome membranes (GM) at several stages of enlargement, (b) glyoxysome membranes of 48-h-old seedlings prepared by diluting glyoxysomes directly in carbonate (GM*), (c) carbonate-washed microsomal membranes (MM), and (d) intact lipid bodies (LB) floated three times. Microsomes were from the 150,000-g, 1-h pellet of a 10,000-g, 20-min supernatant, prepared in potassium phosphate homogenizing medium.

somes). Catalase activity (for glyoxysomes) and protein content were measured. Generally, between 50 and 80% of the catalase activity was recovered in dialyzed/concentrated glyoxysomes and >75% of the total protein was recovered in concentrated samples of both glyoxysomes and mitochondria. In most cases, between 80 and 200 μ g of acceptor (organelle-receiving transported lipid) protein were used in each lipid transfer experiments.

Aliquots of acceptor organelles (routinely 200 μ l) were mixed with aliquots of radiolabeled lipids bodies (containing \sim 100,000 cpm) in microfuge tubes and the volumes of the mixtures were adjusted to 300 μ l with potassium phosphate-containing homogenizing medium. Reaction mixtures were incubated at 37°C (usually 1 h) and terminated by sedimentation of acceptor organelles for 30 min in a microfuge (13,000 g, 4°C). When microsomes were used as the acceptor organelle fraction, the mixture was centrifuged at 180,000 g for 45 min (Airfuge ultracentrifuge Beckman Instruments Inc.) to sediment microsomes. Supernatants, including fat pads, were decanted and, taking care not to disturb the well-packed pellets, the tubes were rinsed three times with 0.5 ml cold potassium phosphate-containing homogenizing medium to remove any lipids adhering to walls. Lipids were extracted from the supernatants plus combined rinses, and radioactivity was measured by liquid scintillation counting or analyzed by TLC and autoradiography as described above (see *Lipid Analyses*). Pellets were resuspended in 0.5 ml potassium phosphate-containing homogenizing medium with vigorous vortexing and transferred to 15-ml glass tubes. The tubes which contained the reaction mixtures were rinsed with 0.3 ml of the same medium which was added to the resuspended pellets. Lipids were extracted and analyzed as described for the supernatant.

Results

Enlargement of Glyoxysomes in Situ

Fig. 1 illustrates the dramatic increase in glyoxysome volume during postgerminative growth. Glyoxysomes are larger, more pleiomorphic structures in parenchyma cells of

Table I. Phospholipid Composition (Weight Percent) of Lipid Bodies, and Carbonate-washed Mitochondrial and Glyoxysomal Membranes Isolated from Cotyledons of 48-h-old Cotton Seedlings

Sample	PG*	PI	PC	PE	PA	CL	Unk	Origin
	%‡							
Lipid bodies	0	34	35	21	4	0	0	6
Mitochondria membranes	1	5	35	32	0	20	0	6
Glyoxysome membranes	13	15	49	12	0	0	4	6

* PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidate; CL, cardiolipin; Unk, unknown.

‡ Percent by weight (μg individual phospholipid/ μg total phospholipid) \times 100. Percentages are averages of 2 two-dimensional TLC separations of 2 independent samples. Lipid bodies were prepared (washed three times by flotation) from 50 cotton seeds. For mitochondria and glyoxysomes, lipids were extracted from membranes prepared from peak organelle fractions pooled from four sucrose density gradients.

48-h-old cotton seedlings (Fig. 1 B) than in parenchyma cells of imbibed seeds (Fig. 1 A). Kuncce et al. (30) demonstrated quantitatively by morphometric analyses that while the glyoxysomes increased in volume sevenfold during this period, the number of glyoxysomes per cell did not change significantly.

Isolated Glyoxysomes

Highly purified fractions of cottonseed glyoxysomes were prepared by sucrose density gradient centrifugation in a vertical rotor in gradients buffered with 100 mM potassium phosphate (pH 7.2). Micrographs of portions of the glyoxysome fractions, and membranes prepared from these organelles, are shown in Fig. 2. Optical microscopy of sections through the entire organelle layer (Fig. 2 A) revealed that the fractions were essentially homogeneous. Electron microscopy revealed that virtually all of the organelles morphologically resembled glyoxysomes (Fig. 2 B). Postembedding immunogold localization of malate synthase in organelles prepared in a similar manner (11) confirmed that these organelles were indeed glyoxysomes. Carbonate-washed membranes prepared from glyoxysomes were essentially free of matrix material (Fig. 2 C).

The average specific activity of isocitrate lyase (a notoriously leaky glyoxysomal matrix enzyme) in glyoxysome fractions prepared under the above conditions was at least twice that of any isolation procedure in which sucrose was used as a gradient medium in our laboratory (e.g., 6, 11). This activity was comparable to specific activities of isocitrate lyase in cottonseed glyoxysome fractions routinely obtained with metrizamide gradients (49). Metrizamide is an isoosmotic density gradient medium which was used as an alternative to sucrose to prepare highly purified glyoxysome fractions from cotton cotyledons (49). Besides indicating that glyoxysomes prepared from cotyledons in sucrose density gradients were highly purified, these data also provided evidence that they were mostly intact.

Lipid Composition of Glyoxysome Membranes

Fig. 3 shows that glyoxysome membranes at various stages of enlargement contain an unusually high proportion of nonpolar lipids, ranging from 62% (by weight) at 4 h to 36% at 48 h. Nonpolar lipids were preferentially removed from

48-h glyoxysome membranes by a two-step procedure (first diluting glyoxysomes in potassium phosphate buffer, and then washing membrane pellets with sodium carbonate), as compared with a one-step procedure (diluting glyoxysomes directly in sodium carbonate; compare 48 h GM with 48 h GM* in Fig. 3), indicating that the association of nonpolar lipids with glyoxysome membranes may be somewhat fragile. As is the case for most biological membranes, considerably less nonpolar lipid (\sim 4% by weight) was in carbonate-washed microsome membranes. Similarly, carbonate-washed mitochondrial membranes exhibited a low proportion of nonpolar lipids (6% by weight, not shown). For comparison, data were presented for cottonseed storage lipid bodies of which nonpolar lipids comprised 98%.

Compositions of individual classes of phospholipids in lipid bodies, and carbonate-washed glyoxysome and mitochondrial membranes are presented in Table I. As expected, PC was the most abundant phospholipid in all of the organelle fractions. PG, PE, and PI were present in nearly equal amounts in glyoxysome membranes. Lipid bodies and, to a lesser degree, glyoxysome membranes, contained a relatively large percentage of PI. Because cardiolipin was restricted to the membranes of the mitochondrial fractions, it appeared that neither lipid bodies nor glyoxysome membrane fractions were contaminated to an appreciable degree with mitochondrial membranes.

Compositions of individual classes of nonpolar lipids in lipid bodies and membranes of glyoxysomes isolated in sucrose gradients or metrizamide gradients are shown in Table II. The kinds or proportions of nonpolar lipids recovered in glyoxysome membranes were not appreciably affected by the type of gradient medium used. While a considerable proportion of the nonpolar lipids in glyoxysome membranes was TAG (13% in sucrose, 16% in metrizamide), FFA was the predominant nonpolar lipid class in glyoxysome membranes (55% in sucrose, 62% in metrizamide). Lipid bodies and glyoxysome membranes shared the same types of nonpolar lipids, but in much different proportions. As expected, virtually all of the nonpolar lipids in lipid bodies were TAG. Neither TAG nor FFA were detected in microsome membranes (not shown); di- and monoacylglycerols, in nearly equal amounts, made up the small percentage (4% by weight, Fig. 3) of nonpolar lipids in microsome membranes.

Fig. 4 is a comparison of gas chromatographic tracings of

Table II. Nonpolar Lipid Composition (Weight Percent) of Lipid Bodies, and Membranes (Carbonate-washed) from Glyoxysomes Isolated in Sucrose or Metrizamide Gradients

Sample	MAG*	DAG	TAG	FFA	Sterols
	%‡				
Lipid bodies	1 \pm 0.2	2 \pm 0.3	97 \pm 2.4	0.4 \pm 0.2	0
Glyoxysomes in sucrose	24 \pm 2.9	9 \pm 1.6	13 \pm 1.7	55 \pm 4.2	1 \pm 0.8
Glyoxysomes in metrizamide	15 \pm 3.4	6 \pm 2.6	16 \pm 2.0	62 \pm 8.6	2 \pm 0.5

* MAG, monoacylglycerols; DAG, diacylglycerols (sn 1,2 and 1,3); TAG, triacylglycerols; FFA, free fatty acids.

‡ Percent by weight (μg individual nonpolar lipid/ μg total nonpolar lipid) \times 100. Means were calculated from values derived from three chromatographic separations of two separate organelle isolations.

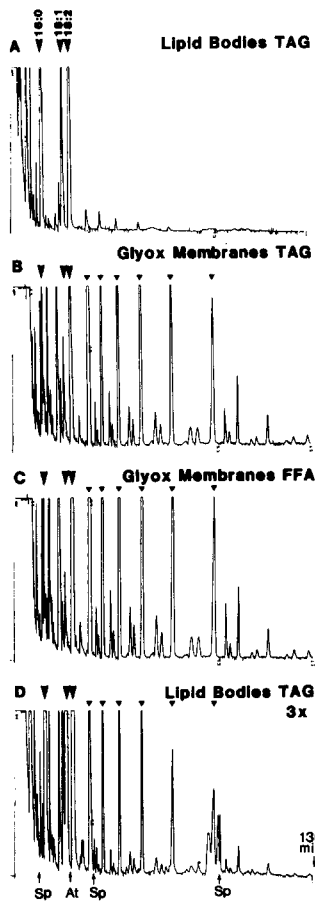


Figure 4. Traces of gas chromatographic separations of fatty acid methyl esters prepared from lipid body TAG (A), glyoxysome membrane TAG (B), glyoxysome membrane FFA (C), and lipid body TAG (D), three times the amount injected for A). Palmitic (16:0), oleic (18:1), and linoleic (18:2) acids were the major fatty acids (large arrowheads) in storage TAG (A and D) while six unidentified fatty acids (small arrowheads) were predominant in glyoxysome membrane TAG and FFA (B and C). *Sp* denotes a twofold reduction in chart speed and *At* signifies a change in attenuation (scale to the right of *At* is twice that to the left).

the separated acyl components of lipid body TAG, glyoxysome membrane TAG, and glyoxysome membrane FFA. Palmitic (16:0, 28% of the total fatty acids), oleic (18:1, 18%), and linoleic (18:2, 34%) acids were the predominant fatty acids of lipid body TAG as expected. All of these fatty acids were detected in glyoxysome membrane TAG and glyoxysome membrane FFA, but in much lower proportions (16:0, 2%, 18:1, <1%, 18:2, 7%). Unexpectedly, six uncommon (and as yet unidentified) fatty acids made up a significant proportion (together 51%) of the fatty acids in both the glyoxysome membrane TAG and glyoxysome membrane FFA fractions. Comparison of the traces in Fig. 4, B and C, with that in Fig. 4 D (three times the amount of material in Fig. 4 A) showed that the predominant fatty acids in glyoxysome membrane TAG and FFA also were present in lipid body TAG, but at a much lower concentration (together ~6%).

Phospholipid Synthesis and Intracellular Transport in Vivo

Fig. 5 shows the results of pulse-chase experiments after applying radiolabeled choline or ethanolamine to cotyledons of 18-h-old cotton seedlings. Applied choline was incorporated into ER (Fig. 5, top) as PC (see Fig. 6 for identification of radiolabeled lipids), whereas ethanolamine was incorporated into ER as PE. Incubation of cotyledons in nonradioactive choline or ethanolamine (2, 12, and 24 h) resulted in a reduction of radiolabeled PC or PE, respectively, in ER fractions with a concomitant increase in radiolabeled PC or PE,

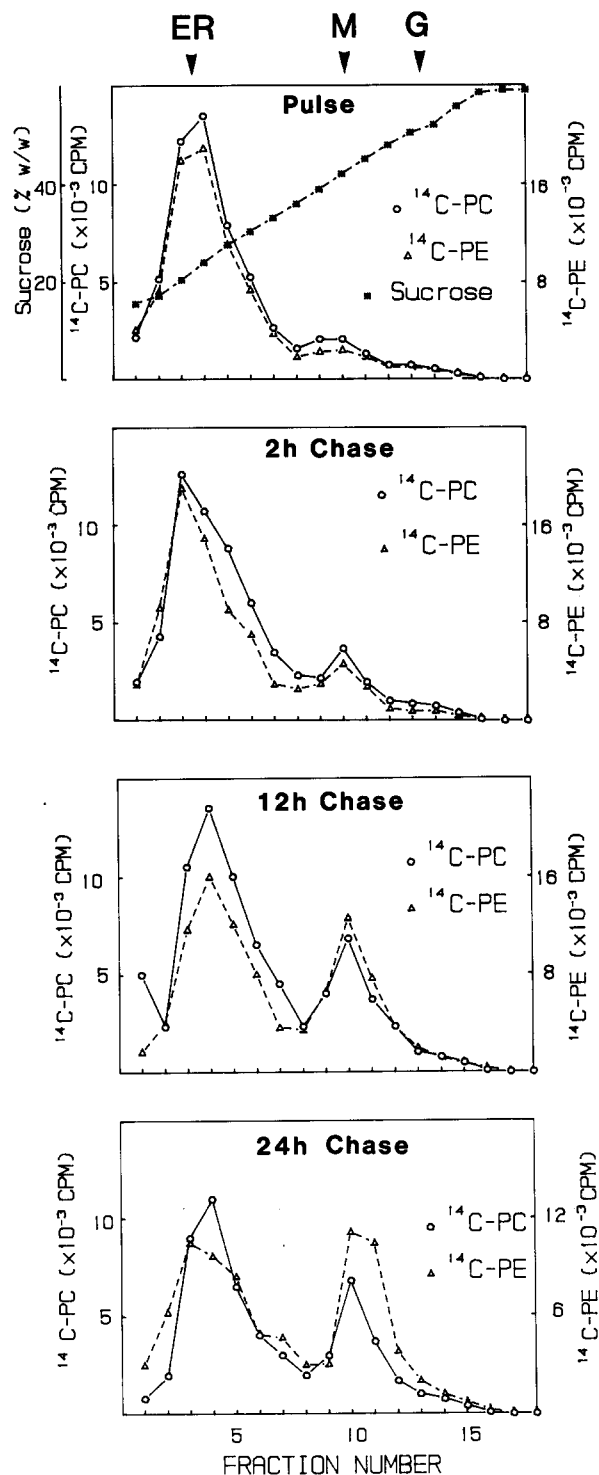


Figure 5. Profiles of radioactive PC and PE in sucrose gradient fractions. Clarified homogenates were fractionated after application of ^{14}C -choline (or ^{14}C -ethanolamine) to 18-h-old cotton cotyledons (1-h pulse for choline, 30-min pulse for ethanolamine), or after a chase period with nonradioactive choline (or nonradioactive ethanolamine). The positions of ER membranes, mitochondria (M), and glyoxysomes (G) were determined in these gradients (denoted at the top by assaying marker enzymes, antimycin A-insensitive NADH cytochrome c reductase, cytochrome c oxidase, and catalase, respectively). Radiolabeled PC and PE were chased into mitochondria fractions but not into glyoxysome fractions.

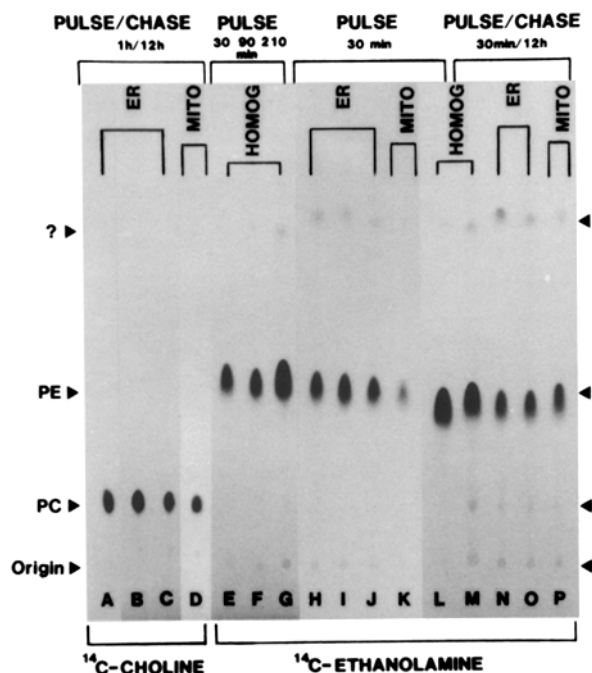


Figure 6. Autoradiograms of radiolabeled lipids extracted from cellular fractions and separated by TLC for identification. Samples for lanes A–D were radiolabeled 1 h with ^{14}C -choline followed by a 12-h chase with nonradioactive choline. Lanes A–C were from fractions 3–5 and lane D was from fraction 10 (see Fig. 5, *third panel*). ^{14}C -Ethanolamine was the radiolabel for samples in lanes E–P. Lanes E–G were lipids from clarified homogenates of cotyledons radiolabeled for an increasing pulse period (30, 90, 210 min). Lanes H–L were lipids from cell fractions prepared after a 30-min pulse. Lanes H–J were from cell fractions 3–5 and lane K was from fraction 10 (see Fig. 5, *top*). Lanes M–P were lipids from cell fractions prepared after a 30-min pulse and 12-h chase with nonradioactive ethanolamine. Lanes N and O were from fractions 4 and 5 and lane P was from fraction 10 (see Fig. 5, *third panel*). Lipids marked with a ? were tentatively identified as *N*-acylPE.

respectively, in mitochondrial fractions. Glyoxysomes did not accumulate ^{14}C -PC or ^{14}C -PE even after the 24-h chase period. Catalase activity increased fourfold (not shown) in cotyledons from 18 to 42 h after imbibition indicating that glyoxysome biogenesis was proceeding normally under these labeling conditions.

The identification of radiolabeled lipids in cellular fractions determined by TLC and autoradiography are revealed in Fig. 6. ^{14}C -PC was the exclusive radioactive lipid product detected in cell fractions in all pulse–chase experiments (up to 210 min pulse, and up to 24 h chase) with ^{14}C -choline. For example, lanes A–D show data when cotyledons were radiolabeled with ^{14}C -choline with a 1-h pulse and 12-h chase.

When cotyledons were pulsed for increasing periods (up to 210 min) with ^{14}C -ethanolamine, ^{14}C -PE was the primary lipid detected in clarified homogenates (Fig. 6, lanes E–G and L). However, some ^{14}C -PC and some ^{14}C -*N*-acylPE (tentative identification, ?, based on cochromatography with *N*-palmitoylPE) also was detected after the 210-min pulse. ^{14}C -PE was the primary radiolabeled lipid product in ER and mitochondria fractions after a 30-min pulse with ^{14}C -ethanolamine (lanes H–K). A small amount of ^{14}C -*N*-acylPE

also was observed in ER, but not in mitochondria fractions. The radiolabeled products in the homogenate (lane M), the ER (lanes N and O), and the mitochondria (lane P) fractions after a 12-h chase were mostly ^{14}C -PE (lanes M–P), and the relative proportions of radioactivity in *N*-acylPE and PC in ER fractions increased with chase time (compare lanes H and I with N and O).

Lipid Transfer In Vivo

Fig. 7 is an electron micrograph of a representative view of the material in the isolated lipid body fractions subjected to lipid analyses and used as donors for in vitro lipid transfer experiments. Lipid body fractions were visibly free from contamination by other cell debris. The dark layer around each lipid body represents the proteinaceous membrane coat composed of oleosins (24) and other lipid body membrane proteins. These lipid bodies were radiolabeled with ^3H -PC or ^3H -TO and served as the “native” donors in lipid transfer experiments.

Fig. 8 shows the relative competence of glyoxysomes, mitochondria, microsomes, and chloroplasts, in acquiring lipids from lipid bodies in vitro. ^3H -PC was transferred to all organelles. Approximately 60% of the ^3H -PC sedimented with membranes (180,000 g, 45 min 4°C) after glyoxysomes or mitochondria were osmotically burst in 100 mM potassium phosphate (pH 7.2). Transfer of ^3H -TO to glyoxysomes was reproducibly established, whereas transfer of ^3H -TO to other organelles was insignificant. Nearly 100% of the radiolabeled TO transferred to glyoxysomes remained associated with membranes after osmotic disruption and sedimentation of membranes. Simultaneous experimental controls without acceptor organelles (not shown) yielded low amounts of radioactivity (~ 400 cpm) remaining in these control tubes after removal of the supernatant. This indicated that contami-

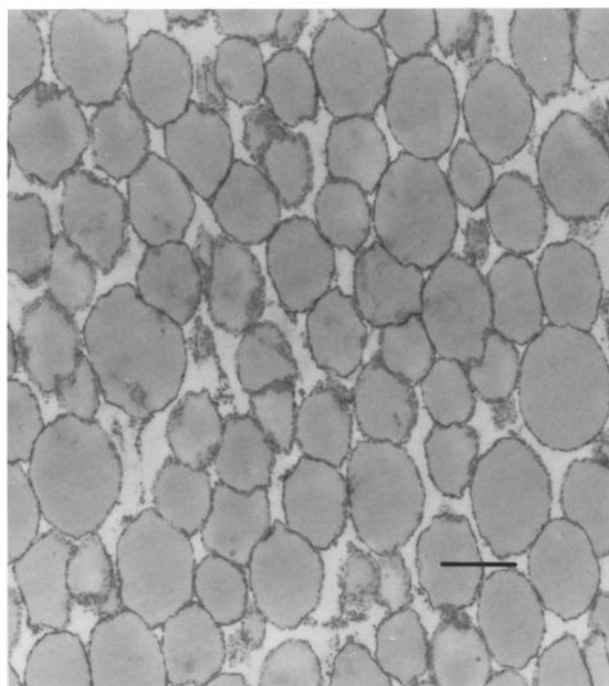


Figure 7. Electron micrograph of purified lipid bodies (floated three times) from cotyledons of 24-h-old cotton seedlings. Bar, 1.0 μm .

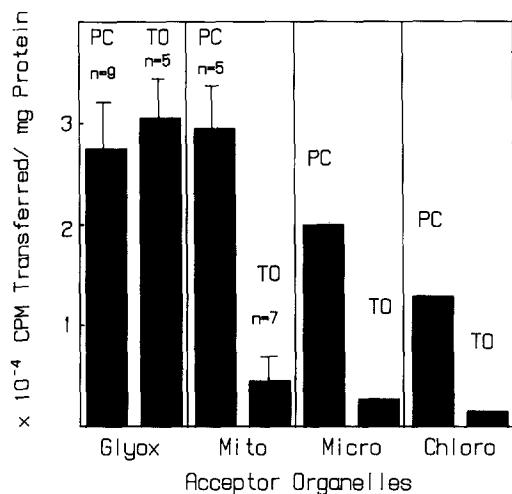


Figure 8. Amounts of $^3\text{H-PC}$ or $^3\text{H-TO}$ transferred in vitro from radiolabeled lipid bodies to glyoxysomes, mitochondria, microsomes (prepared as described in legend for Fig. 3), or chloroplasts (from a 5,720-g, 5-min pellet of a 640-g, 10-min supernatant of homogenized cotyledons grown 2 d in the dark followed by 3 d in continuous light). Radiolabeled lipid bodies were mixed with isolated organelles and incubated 1 h at 37°C; acceptor organelles were sedimented (donor lipid bodies floated) at 13,000 g for 30 min (except for microsomes pelleted at 180,000 g, 45 min), and radioactivity was measured in lipid extracts. Values for glyoxysomes and mitochondria are means with standard deviation (n = number of independent experiments). Values for microsomes and chloroplasts are the average of two transfers.

nation of acceptor organelle fractions by sedimentation of radiolabeled lipid bodies (because of wall effects) did not account for the observed radioactivity in the acceptor organelle fractions.

Fig. 9 compares the effectiveness of lipid bodies, with and without associated proteins, as donors. When synthetic lipid bodies (constructed in vitro from lipids extracted from isolated lipid bodies) were tested for transport competence, the amounts of radiolabeled lipids (TO and PC) associated with

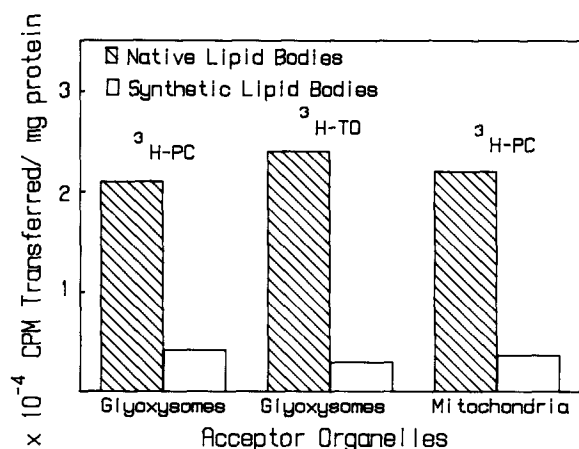


Figure 9. Amounts of radiolabeled TO or PC transferred in vitro from native (with associated protein, same as for Fig. 8) or synthetic (without associated protein) lipid bodies to glyoxysomes or mitochondria. Values are the average of two independent experiments.

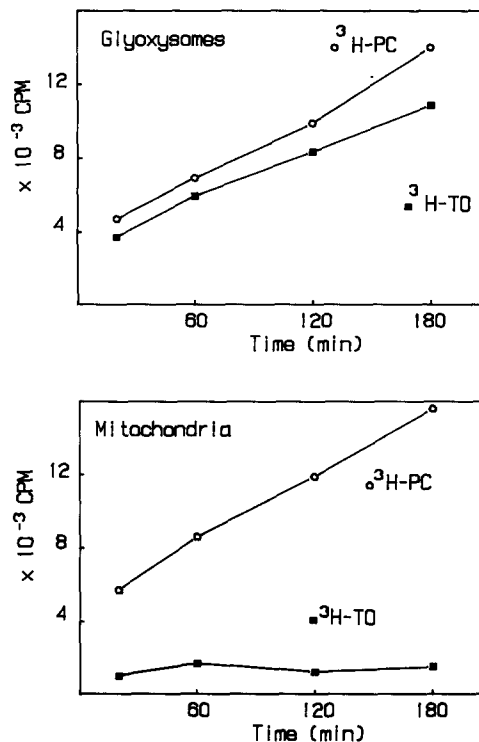


Figure 10. Time courses of transfer of radiolabeled PC and TO in vitro from lipid bodies to glyoxysomes (185 μg protein) or mitochondria (240 μg protein). Data are representative of three independent experiments.

glyoxysomes and mitochondria were substantially lower compared with the amounts of lipids transferred from native lipid bodies (with associated proteins as for Fig. 8). Simultaneous experimental controls with native lipid bodies and without acceptor organelles yielded low amounts of radioactivity (cpm were similar to amounts when synthetic lipid bodies were used as the donor, Fig. 9) associated with the tubes after removal of the supernatant. This suggested that the radioactivity associated with glyoxysomes and mitochondria after incubation with radiolabeled synthetic lipid bodies was not a result of bona fide transfer, but likely was due to contamination of pellets by radiolabeled lipids or by radiolabeled lipids adhering to the wall of the tube after removal of the supernatant.

Time courses of the transfer of $^3\text{H-PC}$ and $^3\text{H-TO}$ from lipid bodies to glyoxysomes and mitochondria are shown in Fig. 10. Transfer of $^3\text{H-PC}$ and $^3\text{H-TO}$ increased linearly between 20 and 180 min when glyoxysomes were used as the acceptor organelle fraction (top). Transfer of $^3\text{H-PC}$, but not $^3\text{H-TO}$, increased linearly during this time frame when mitochondria were used as the acceptor organelle fraction (bottom).

Fig. 11 shows the relationship between the amount of transferred $^3\text{H-TO}$ and the amount of acceptor organelle protein. The transfer of $^3\text{H-TO}$ from lipid bodies to glyoxysomes increased linearly with the increasing amounts of glyoxysomal protein tested, while the amount of $^3\text{H-TO}$ in mitochondrial fractions did not change relative to the amount of mitochondrial protein. In contrast, transfer of $^3\text{H-PC}$ from lipid bodies to both glyoxysomes and mito-

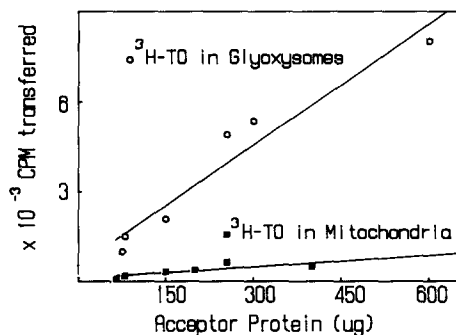


Figure 11. Influence of the amount of acceptor organelle protein on the transfer in vitro of $^3\text{H-TO}$ from lipid bodies to glyoxysomes or mitochondria. Data points are single values from two independent experiments for glyoxysomes and three independent experiments for mitochondria.

chondria increased linearly with the amount of glyoxysomal and mitochondrial protein (not shown). When twice the amount of radiolabeled lipid bodies was added to a transfer assay mixture, the amount of transferred radiolabeled lipid did not change for a given amount of acceptor organelle protein (not shown).

Identities of radiolabeled lipids in organelle fractions after a 1-h incubation in the lipid transfer assay reaction mixture are revealed in Fig. 12. PC was the only radiolabeled lipid transferred to glyoxysomes and mitochondria from lipid bodies radiolabeled with $^3\text{H-PC}$ (Fig. 12 A). Interestingly, the TO transferred from lipid bodies to glyoxysomes was partially hydrolyzed to FFA, while TO remaining in lipid bodies was not (B). Hydrolysis of TO in glyoxysomes appeared to be relatively specific because the acyl groups of PC were not hydrolyzed (A).

Discussion

Nonpolar Lipids in Glyoxysome Membranes

Glyoxysomes in cotyledons of cotton seedlings enlarge dramatically during the first 48 h of postgerminative growth (see Fig. 1, see also reference 30). Because they do not synthesize their own major phospholipids (PC and PE, reference 10) a significant amount of membrane lipid must be delivered to glyoxysomes to accommodate membrane expansion. An unexpected result was that highly purified glyoxysome membranes (Fig. 2) were composed of 36–62% (by weight) nonpolar lipids (Fig. 3). FFA in 48-h glyoxysome membranes comprised ~20% of the total membrane lipid (57% of 36%, from Fig. 3 and Table II). The abundance of six uncommon (and unidentified) fatty acids in both TAG and FFA of glyoxysome membranes (Fig. 4) was intriguing, especially since they also were in TAG of lipid bodies, albeit at a low concentration.

To our knowledge, no biological membrane has been described with such a high proportion of nonpolar lipids. It was unlikely that the nonpolar lipids in glyoxysome membranes were from contaminating lipid bodies because the proportions of nonpolar lipid classes (and the proportions of acyl groups within these classes) were much different in these two organelles (Table II, Fig. 4). Also lipid bodies were not observed in micrographs (optical or electron) of purified glyox-

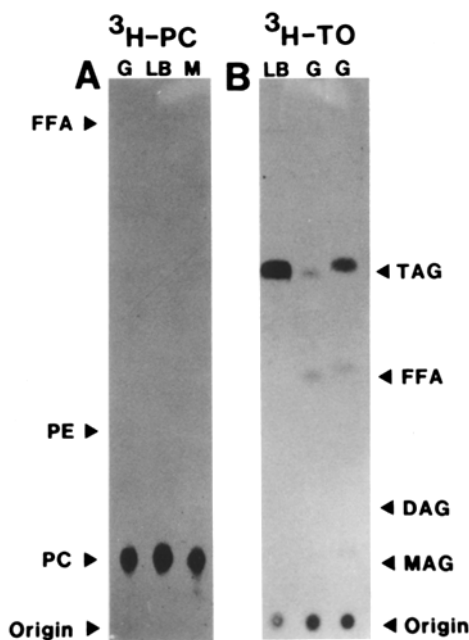


Figure 12. Autoradiograms of TLC separations of lipids extracted from organelle fractions after in vitro transfer of $^3\text{H-PC}$ (A) or $^3\text{H-TO}$ (B). Both plates were developed in hexane/ether/formate (80:20:2). For A, the phospholipids were then separated in chloroform/methanol/water (80:35:3). Plates were sprayed with EN³-HANCE before exposure of film (14 d at -85°C). PC was the only radiolabeled lipid transferred to glyoxysomes (G) and mitochondria (M), and was the only radiolabeled lipid that remained in lipid bodies (LB) when donor lipid bodies contained $^3\text{H-PC}$. When the donor lipid body fraction was radiolabeled with $^3\text{H-TO}$, radioactivity remaining in lipid bodies after the transfer reaction comigrated exclusively with TAG. Both TAG and FFA were detected in glyoxysomes after transfer, indicating that TO was transferred to glyoxysomes before hydrolysis. In B, the two lanes labeled G were glyoxysome lipids from two independent transfer experiments.

ysomes or glyoxysome membranes (Fig. 2). Furthermore, nonspecific hydrolysis of acylated lipids likely was not responsible for the high percentage of FFA in glyoxysome membranes for at least two reasons. First, the membrane preparation scheme or lipid extraction procedure did not liberate FFA because hydrolysis of mitochondrial or microsomal membrane lipids was not observed. Second, a nonspecific acylhydrolase was not active in glyoxysome membranes because radiolabeled PC transferred to glyoxysomes was not hydrolyzed during or after the in vitro lipid transfer assay (Fig. 12, A). Most likely, the FFA arose by specific hydrolysis of TAG incorporated into the glyoxysome membrane (Fig. 12 B; see also later discussion).

The nonpolar lipid composition of membranes typically is not analyzed because, with the exception of sterols, these lipids are not considered as important or prevalent membrane components. Recently, however, evidence was presented which indicated that neutral lipid domains existed in plasma membranes of activated, stimulated, or transformed cells (36). Microdomains of primarily TAG (6% of the total membrane lipids) were proposed to be arranged into droplets intercalated within the hydrophobic phase of the lipid bilayer and covered by phospholipids (36). It remains to be tested whether the molecular arrangement of nonpolar lipids in glyoxysome membranes is consistent with this model.

Some data exist on the nonpolar lipid compositions of membranes surrounding peroxisomes isolated from two different organisms (castor bean and the yeast, *Candida tropicalis*). Endosperm glyoxysome membranes contained 10% by weight (estimated from the mol% values reported) nonpolar lipids, 60% of which were FFA (12, 17). More than 95% of the FFA were palmitic, stearic, oleic, and linoleic acids. A smaller amount of diacylglycerols (0.7 mol%), TAG (0.6%), and sterols (0.8%) also were reported. Nonpolar lipids in the yeast peroxisome membranes were not quantified, but were identified as mostly sterols, with some FFA (40).

Peroxisomes oxidize fatty acids in virtually all organisms examined (2, 22, 25, 29, 31, 46, 47, 52), hence the appearance of FFA in cottonseed glyoxysome membranes was not altogether surprising. One obvious function of these fatty acids would be to serve as substrates for the β -oxidation enzymes located in the matrix of cottonseed (6) and other peroxisomes (22, 29). If this were the case, then the three common FFA detected at low concentration in the glyoxysome membrane may simply be in a state of flux through the membrane. A more speculative function for the unusual fatty acids sequestered at a higher concentration in glyoxysome membranes might be their specific involvement in altering membrane fluidity, thereby facilitating membrane expansion. A third possible function for the glyoxysome membrane FFA is formation of pores for the exchange of cofactors and metabolites (51). Reports exist which demonstrate increased permeability of membrane vesicles caused by formation of pores at elevated concentrations of FFA (7). Permeability of peroxisome membranes increased when livers of mice were treated with proliferators (15). This was believed to result from an increase in lysophosphatidylcholine and a decrease in PC. The increase in amount of FFA that would obligatorily arise from the hydrolysis of PC to form lysoPC was not addressed by the authors. Studies are planned to identify the unusual glyoxysomal membrane FFA so their actual role in glyoxysome membrane function can be explored.

Glyoxysome Membrane PC and PE Are Not Transported from ER

Models describing the biogenesis of peroxisomes often include an aspect of membrane biogenesis whereby newly synthesized peroxisome membrane lipids are derived from ER (21, 25, 31, 32, 53). Evidence supporting this concept is mostly indirect; i.e., the ER is the primary cellular location of phospholipid-synthesizing enzymes (for PC in particular), whereas peroxisomes do not appear to synthesize their own membrane phospholipids (4, 10, 25, 33, 46). Direct evidence supporting ER as the source of peroxisome membrane lipids is meager. For example, the most current model for glyoxysome biogenesis in castor bean endosperm (21, 32) relies on the de novo proliferation of glyoxysomes by budding from segments of ER from which membrane lipid is contributed directly. Data from only one paper support this portion of the model (28). Differential rates of decline in radiolabeling of ER and glyoxysome fractions were interpreted to indicate that radiolabeled PC was transported from ER to glyoxysomes in vivo. There are no other data from any other organism that demonstrate direct transfer of membrane lipids from ER to peroxisomes. Results from our pulse-chase radiolabeling experiments (Figs. 5 and 6) demon-

strated that PC and PE were transferred from ER to mitochondria, but not from ER to enlarging glyoxysomes. In earlier work, we showed that enlarging glyoxysomes were incapable of synthesizing their own PC and PE (10). Therefore, peroxisomes in general, and more specifically enlarging cottonseed glyoxysomes, may acquire their membrane phospholipids from an alternative cellular source.

Glyoxysome Membrane Lipids Are Derived from Lipid Bodies

Several lines of evidence suggested that lipid bodies could be the source of membrane lipids for enlarging cottonseed glyoxysomes. First, from our analyses of lipid classes, it was apparent that components of lipid bodies were similar to those in glyoxysome membranes. Second, lipid bodies and glyoxysomes are in close physical proximity during mobilization of reserve oil (3, 25, 47). Third, there is a paucity of evidence for ER being the source of phospholipid (PC and PE) for endosperm or cotyledon glyoxysome membranes (see above).

To test our hypothesis, an in vitro lipid transfer assay system was developed. The direct transfer of ^3H -PC and ^3H -TO from lipid bodies to glyoxysomes (Figs. 8–12) suggested that this phenomenon could indeed occur in vivo. Radiolabeling experiments in vivo were not possible because lipid bodies are synthesized during seed development (in the cotton boll); consequently, they could not be radiolabeled to a sufficiently high radiospecific activity during seed germination to determine their role as possible lipid donors. In vitro, all organelle fractions tested acquired PC whereas only glyoxysomes acquired TO (Fig. 8). Control experiments with synthetic lipid bodies (Fig. 9) also helped show that the apparent transfer of lipids was not attributable to nonspecific adsorption or adherence of lipids to the surface of glyoxysomes or mitochondria. Synthetic lipid body preparations were not floated after radiolabeling, hence ^3H -lipids not incorporated into lipid bodies (e.g., PC micelles and liposomes) were free to fuse (bind) nonspecifically with acceptor membranes; this did not occur to an appreciable extent. The incorporation of transferred radiolabeled lipids into membranes also argued against nonspecific adsorption by lipid bodies.

Few details are known about the actual transfer of reserve lipid to glyoxysomes for β -oxidation (24, 29, 35, 47). The subcellular location of the lipase(s) involved in TAG hydrolysis has been the subject of much controversy and is apparently species specific (24, 26, 29, 47). In cotton cotyledons, the available data indicate that the TAG lipase is mostly cytosolic and is translocated to lipid bodies for oil mobilization during postgerminative growth (24). This implies that fatty acids are released from lipid bodies for transport to glyoxysomes. Unlike the situation for mammalian mitochondria, β -oxidation of fatty acids in peroxisomes is carnitine independent (22, 29), implying that the peroxisome matrix is readily accessible to FFA. In our work, radiolabeled FFA were detected in glyoxysome membranes after transport of TO (Fig. 12 B), indicating that TAG was the molecular species transferred to glyoxysome membranes, then hydrolyzed presumably by a glyoxysomal TAG lipase. To date only three seeds (castor bean, peanut, soybean) have been identified which contain true lipases (capable of hydrolyzing TAG) in their glyoxysomes (47). Only the lipase in soybean glyoxysomes

is considered to be the major lipolytic enzyme responsible for oil mobilization (47). In most oilseeds, lipases associated with the lipid bodies are the major (if not exclusive) lipolytic enzymes (24, 26). The carnitine independence of peroxisomal fatty acid β -oxidation may have evolved to accommodate multiple routes of fatty acid entry (free form or acylated as TAG) into peroxisomes. A possible explanation for the low or nonexistent activity of TAG lipases reported for cottonseed, or other oilseed, glyoxysomes is as follows. Characteristically, lipase assays are conducted with emulsions of TAG as substrate. Our emulsions (synthetic lipid bodies) proved to be poor donors for the transfer of TO to glyoxysomes (Fig. 9). Perhaps other researchers were unable to detect TAG hydrolysis with glyoxysomes because TAG transport was not reconstituted. We also measured lipase activity in the cytosolic (soluble) fractions of cotton cotyledon homogenates (with our native lipid bodies as substrate), but the activity(ies) was nonspecific; i.e., PC was hydrolyzed as well as TO, and the specific activity in the cytosol (51.5 nmol oleic acid released/h per mg protein) was lower than that measured in glyoxysomes (101 nmol oleic acid released/h per mg organelle protein). We believe that we have essentially reconstituted lipid mobilization process in seeds.

Unlike many other *in vitro* lipid transfer systems (27 and references therein; 34), transport and incorporation of lipid into glyoxysome membranes was accomplished without addition of any other components such as cytosolic fractions, purified lipid transfer proteins, cofactors, high energy nucleotides, etc. An inconsistent stimulatory effect on transfer was obtained by adding of cytosol (150,000-g supernatant) to our transfer assay mixture. In this regard, it is worth noting that transfer of radiolabeled PC or radiolabeled PE from ER membranes (radiolabeled by incubating ^{14}C -CDP-choline or ^{14}C -CDPethanolamine with microsomes; reference 10) to glyoxysomes could not be demonstrated *in vitro*, even with added ATP and/or cytosolic fractions (data not shown).

Experiments in this paper were not designed to directly address the mechanism(s) of lipid transfer between lipid bodies and glyoxysomes. Some general inferences, however, can be made. Transfer was nearly abolished in the absence of proteins normally associated with lipid bodies (Fig. 9, compare native lipid bodies with synthetic lipid bodies). The transport competence of lipid bodies was greatly reduced when lipid bodies had been stored at -20°C for as little as 16 h, suggesting that freeze-thawing disrupted the structural integrity of the transport machinery. The major lipid body membrane proteins, termed oleosins by Huang et al. (26), appear to be highly conserved and ubiquitous in lipid bodies of diverse species (26). Perhaps the oleosins themselves catalyze lipid transfer or, alternatively, serve as receptors in recognition/docking of the two organelles such that transfer can occur.

Fig. 13 is an interpretive summary of our results. Lipids (polar and nonpolar) are transported directly from lipid bodies and incorporated into membranes of glyoxysomes to accommodate postgerminative organelle enlargement and membrane differentiation. This occurs concomitant with the posttranslational accumulation of glyoxysomal matrix enzymes and mobilization of oil reserves. Newly synthesized PC and PE are not transferred to enlarging glyoxysomes from ER. It remains to be tested whether this model, or variations thereof, apply to membrane biogenesis in other sys-

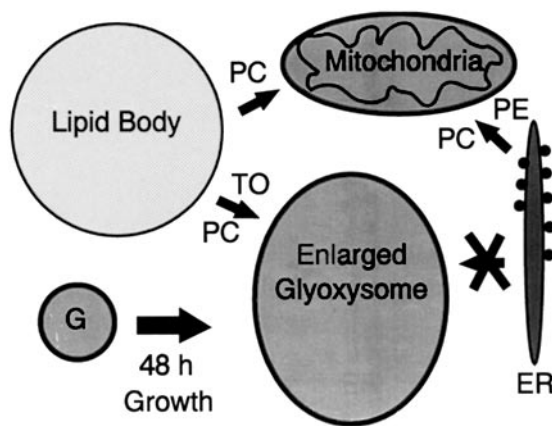


Figure 13. Proposed scheme depicting intracellular lipid trafficking in cotyledons of cotton seedlings during glyoxysome enlargement and storage oil mobilization. Preexisting glyoxysomes (*G*) in imbibed seeds enlarge dramatically during postgerminative growth. PC and PE are transferred from ER to mitochondria, but not to enlarging glyoxysomes. Instead, nonpolar lipids (*TO*) and phospholipids (*PC*) are supplied by lipid bodies which are catabolized as a heterotrophic carbon source during the period of glyoxysome enlargement.

tems where peroxisome enlargement (elaboration) is documented, e.g., other cotyledons, yeasts, and mammalian liver. It is clear that more direct experimental evidence is needed to support models in which ER is presumed to be the source of peroxisome membrane lipids.

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