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 imbition (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, 1H-triolein (TO) and 1H-PC were indeed transferred from lipid bodies to glyoxysomes. 1H-PC, but not 1H-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. 1H-TO was transferred to and incorporated into glyoxysome membranes, and then hydrolyzed to free fatty acids. 1H-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 enlarge in volume sevenfold within 48 h after seed imbition (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 peroxisomal biogenesis that consider the origin of peroxisomal 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) 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 ethanolaminephosphotransferase 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.

I. 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, hemate, diethyl ether, tetrahydrofuran, formic acid (88%), glacial acetic acid, ammonium hydroxide, chloroform, methanol, and sucrose (ribonucleic 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), c-phosphatidy(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-phosphatidyl-9,10-3H(N)] PC, [1-c-dipalmityl (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 in 15-cm glass petri plates for germination and growth in the dark at 30°C. For radiolabeling experiments, imbibed seeds were first decorticated, 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 150 seedlings were adequate. The homogenate was filtered through a multiple layer of Miracloth (pre-soaked with homogenizing medium) and centrifuged (10 min at 10,000 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-
or 60 min, 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 re-suspended 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 (2:1, vol/vol) to yield a final ratio of chloroform/methanol/water (2:2:0.8) (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 Tablettop centrifuge, IFE, Needham Heights, MA). The lower chloroform phase was washed two additional times with 2 ml of 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 mM sucrose) was layered over the condensed 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 1.5 ml corex tubes. [H]TO (10 μCi) or [H]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 [H]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 (flotted three times) as described above (see Preparation of Organelle Membranes), evaporated to dryness under N2, dissolved in 1 ml 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 (trisglycerol [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% (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). Catalase cytochemistry was performed on fixed tissue segments (~1 mm³) using a 1-min preincubation at 30°C with 1.0% (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 centrifuged (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) nage. 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 for encasing the pellet in a drop of 5% nage. 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 mm³) in 5% nage. The lipid bodies were then maintained for TEM as described above for glyoxysomes.

**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 10 x 20 cm silica plates (Chromarods-SII, stored in 6 M HNO3). Lipid samples (in chloroform (1 μl)) were spotted onto pads (previously washed twice in water, dried 1 h at 120°C), then equilibrated at 52% relative humidity (by 15-min incubation over saturated Na2C2O4 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 (80:35:5, 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 PI, PI, and PE overlapped somewhat in the solvent system used for Iatroscan TLC/FID. Therefore, two-dimensional TLC was used to obtain accurate locations of phospholipid classes in organelle fractions. Nonpolar lipids were first separated from polar lipids by spotting samples (in ~40 μl chloroform) onto silica G plates (0.25-mm silica gel G, 10 x 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
paper in 150 x 25 mm plastic petri plates containing 8 ml ultrapure water.

Incorporation of (methyl-1°C) choline chloride and (1,2 ethanolamine-1°C)

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, 6000–8000 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 μm; (B) 3 μm; (C) 0.5 μm.

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 100,000-g, 20-min supernatant, prepared in potassium phosphate homogenizing medium.

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
of 48-h-old Cotton Seedlings

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

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

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.

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

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

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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, resulting in an increase of PC in ER fractions and a decrease in PC in PE fractions. The positions of ER membranes, mitochondria (M), and glyoxysomes (G) were determined in these gradients (denoted at the top by assaying marker enzymes, antimony 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. 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 $^{3}$H-PC or $^{3}$HTO 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. $^{3}$H-PC was transferred to all organelles. Approximately 60% of the $^{3}$H-PC sedimented with membranes (180,000 g, 40 min 4°C) after glyoxysomes or mitochondria were osmotically burst in 100 mM potassium phosphate (pH 7.2). Transfer of $^{3}$HTO to glyoxysomes was reproducibly established, whereas transfer of $^{3}$HTO 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-
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 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 $^3$H-PC and $^3$H-TO from lipid bodies to glyoxysomes and mitochondria are shown in Fig. 10. Transfer of $^3$H-PC and $^3$H-TO increased linearly between 20 and 180 min when glyoxysomes were used as the acceptor organelle fraction (top). Transfer of $^3$H-PC, but not $^3$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 $^3$H-PC and the amount of acceptor organelle protein. The transfer of $^3$H-TO from lipid bodies to glyoxysomes increased linearly with the increasing amounts of glyoxysomal protein tested, while the amount of $^3$H-TO in mitochondrial fractions did not change relative to the amount of mitochondrial protein. In contrast, transfer of $^3$H-PC from lipid bodies to both glyoxysomes and mito-
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 I). 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 glyoxysomes 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 cotonseed (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 glyoxysomes 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) demonstrated 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 organellar 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 cotton seed, 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 14C-CDP-choline or 14C-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 systems 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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