Plant Physiol. (1973) 51, 61–65

The Origin and Turnover of Organelle Membranes in Castor Bean Endosperm[1]

Received for publication May 4, 1972

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ABSTRACT

The origin and turnover of organelle membranes in castor bean (*Ricinus communis* L. var. Hale) endosperm was examined using choline-^{14}C as a phospholipid precursor. On sucrose gradients three major particulate fractions were separated; a light membranous fraction (density 1.11–1.13 gram per cm^3), the mitochondria (1.18 gram per cm^3), and the glyoxysomes (1.24 gram per cm^3). Choline-^{14}C was readily incorporated into lecithin in all three particulate fractions, but the light membranous fraction became labeled first. Incorporation continued into all three fractions for 6 hours, at which time the available choline-^{14}C had been completely used. Subsequently, ^{14}C was lost from the three components at distinctly different rates. When an excess of unlabeled choline was added after 1 hour (pulse-chase experiment), incorporation of choline-^{14}C into glyoxysomes and mitochondria continued for three hours, but at a diminishing rate. This was followed by a period in which the ^{14}C content of the mitochondria declined at a rate expected, if the half life of lecithin in the membrane were about 50 hours and that of the glyoxysomes 10 hours. These values are close to those calculated from the experiments in which no chase was used. The labeling in the light membrane fraction behaved differently from that of the mitochondria and glyoxysomes following the chase of unlabeled choline. Incorporation continued for only 1 additional hour, and then the ^{14}C content declined sharply in the subsequent 4 hours. The early kinetics and subsequent interrelationships are those expected if the lecithin in the membranes of mitochondria and glyoxysomes originates in components of the light membrane fraction.

A striking increase in organelles housing enzymes of the gluconeogenic pathway occurs during the onset of fat breakdown in seedlings (4, 8, 11–13, 17, 20, 21). In the endosperm of castor bean seeds germinating at 30 C, the numbers of glyoxysomes and mitochondria increase during the first 5 days and decline when fat utilization is complete (8). The mechanism of biogenesis, maintenance, and destruction of these organelles is of interest. One approach is to examine the turnover of proteins and individual enzymes (9, 16). Another consideration is the behavior of the membranes surrounding the organelles. The effectiveness of choline as a precursor of membrane constituents has already been demonstrated in several plant systems (3, 7, 14).

Here we examine the origin and turnover of organelle membranes as revealed by the incorporation of choline-^{14}C into lecithin.

MATERIALS AND METHODS

Plant Material. Seeds of castor bean (*Ricinus communis* L. var. Hale) were soaked in running water for 1 day and grown in moist vermiculite in darkness at 30 C. Seedlings of particular ages were carefully chosen for uniformity, and the seeds were detached at the hypocotyl. The testae were removed, and the seeds were surface sterilized by immersion for 10 sec in 30% (v/v) Clorox solution and 30 sec in 5% Clorox. After three 1-min rinses in sterile distilled water, endosperm halves (10 per treatment) were removed aseptically and placed abaxial surface down on sterile 1% agar in Petri dishes.

Application of Choline-^{14}C. Choline chloride (methyl ^{14}C) from New England Nuclear was used. Samples of 2.5 μl, containing 0.025 μmoles of choline and 0.25 μc of ^{14}C in ethanol were applied to the inner surfaces of each half endosperm (18). The covered dishes were incubated at 30 C in darkness for appropriate periods. In pulse-chase experiments 2.5 μl of unlabeled choline chloride (1.25 M in ethanol) were applied directly to the endosperm halves 1 hr after the application of choline-^{14}C.

Preparation of Extracts. After incubation, the endosperm halves were homogenized by chopping for 15 min with a stainless steel razor blade in 6 ml of grinding medium in a Petri dish. The grinding medium contained 0.56 M sucrose, 0.15 M tricine, pH 7.5, 10 mM dithiothreitol, 10 mM KCl, 1 mM $MgCl_2$, and 1 mM EDTA, pH 7.5. The homogenate was filtered through two layers of nylon cloth, and the residue was washed twice with 2 ml of grinding medium. The combined filtrates were centrifuged at 270g for 10 min to remove cell debris. The fat layer was removed, and the supernatant (crude homogenate) was decanted. All procedures were carried out at 0 to 4 C.

Isolation of Crude Particulate Fractions. To obtain the total particulate fraction, the crude homogenate was centrifuged at 150,000g for 1 hr. The pellet was suspended in 2 ml of grinding medium.

Sucrose Gradient Separations. Four ml of the crude homogenate were layered on to 26 ml of a linear gradient of sucrose 32 to 60% (w/w) over a 2-ml cushion of 60% sucrose in a 1 inch × 3½ inches (38.5 ml) polyallomer tube. The sucrose solutions contained 10 mM triethanolamine, 10 mM potassium acetate, and 1 mM $MgCl_2$ at pH 7.5. After centrifuging at 53,000g for 4 hr at 2 C in the SW 27 rotor on a Beckman L2-65B centrifuge, three major protein bands were visible. The lowermost, at density 1.24 g per cm^3, was the glyoxysome fraction and that at 1.18 g per cm^3 was mitochondrial. The lightest

[1] This work was supported by National Science Foundation Grant GB 24961.

fraction, in the range of density 1.11 to 1.13 g per cm^3, will be designated the light membranous fraction. For better separation of this fraction from the soluble components in the kinetic experiments, a second 4-ml sample of the crude homogenate was layered on to 26 ml of a linear gradient of 20 to 40% sucrose over a 60% sucrose cushion and centrifuged as described above. Following centrifugation, 0.6-ml fractions were collected across the gradients using an Isco density gradient fractionator, Model 640.

^{14}C Assays. Portions (50, 100, and 200 μl) of each of the fractions were added to 10 ml of scintillation fluid (100 g of naphthalene and 5 g of 2,5-diphenyloxazole in 1 liter of dioxane) in counting vials. ^{14}C was assayed in a Beckman LS 200B instrument where the counting efficiency was 80%. Radioactivity was shown to be proportional to the amount of the cell fraction assayed.

Extraction of Lipids from Crude Particulate Fraction. The total particulate fraction from five endosperm halves which had been exposed to choline-^{14}C for 24 hr was suspended in 3 ml of grinding medium. Lipid was extracted in 15 ml of chloroform-methanol (1:2 v/v) after the method of Allen and Good (1). Polar lipids were separated on Eastman silica gel by thin layer chromatography (1). Lipid-containing areas were located by exposing the chromatograms to iodine vapor and phosphorus-containing areas by spraying with molybdate reagent (1).

Enzyme Assays. Catalase, isocitrate lyase, and fumarase were assayed as described previously (6).

Other Methods. Sucrose concentration was determined refractometrically, and nucleic acid was measured by the procedure of Warburg and Christian. (22).

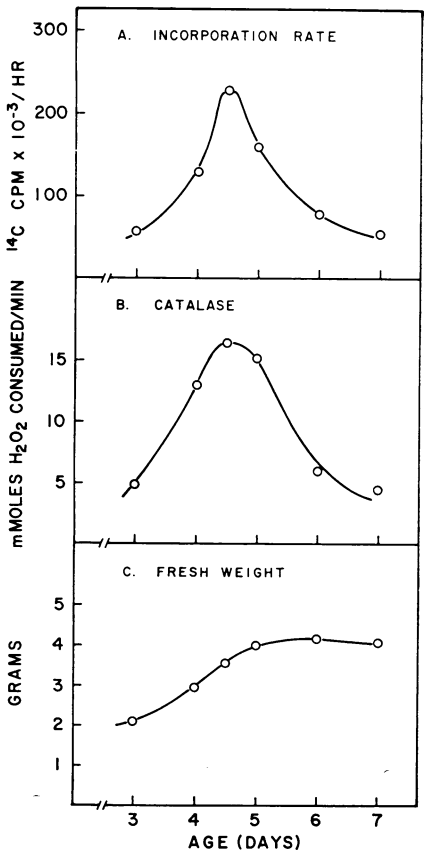

FIG. 1. Effect of age on the rate of choline-^{14}C incorporation into particulate fractions (A), catalase activity (B), and fresh weight (C) of 10 castor bean endosperm halves.

Table I. *Identification of ^{14}C Remaining in the Soluble Fraction of Castor Bean Endosperm Cells*

^{14}C remaining in the supernatant fraction of castor bean endosperm cells when incorporation of choline-^{14}C into particulate cell fractions had ceased was adsorbed onto a column (1 × 5 cm) of Dowex 50 (H^+ form), and its elution behavior was compared with that of choline-^{14}C.

	Supernatant	Choline-^{14}C
	cpm	
Radioactivity added to column	256,300	232,240
Radioactivity eluted with 10 ml of		
Water	0	0
2 N NH_4OH	57,000	0
Water	0	0
3 N HCl	220,000	250,000

RESULTS

Choline-^{14}C Incorporation during Seedling Development. Choline-^{14}C applied to the excised endosperm halves rapidly permeates the whole tissue and is actively incorporated into the total particulate fraction. As shown in Figure 1A this capacity increases strikingly during the early stages of development and declines after day 5. The endosperm is expanding during the first 5 days (Fig. 1C), and it is during this time that a net increase in mitochondria and glyoxysomes occurs (8). The curve for total catalase, a glyoxysome marker (Fig. 1B), is an indicator of the cellular content of these organelles and parallels closely the changes in rates of incorporation of choline-^{14}C. Endosperm tissue from 4-day seedlings was chosen for subsequent experiments. Excised seeds weighing about 0.6 g were selected, and the kinetics of choline-^{14}C incorporation into the particulate fraction was examined. Incorporation began at once and continued for 6 hr, after which no further increase occurred. At 6 hr approximately 50% of the added ^{14}C was recovered in this fraction. The supernatant solution was found to contain no fumarase activity and only 2% of the isocitrate lyase, showing that virtually no breakage of mitochondria or glyoxysomes had occurred during grinding and centrifugation.

Analysis of Lipid Products of Choline-^{14}C Incorporation. The lipid components of the particulate fraction obtained after a 24-hr incubation with choline-^{14}C were extracted in chloroform-methanol and examined chromatographically (see "Materials and Methods"). Over 90% of the ^{14}C extracted had the same R_F as authentic lecithin in chloroform-methanol-water (65:25:4 v:v:v) and chloroform-methanol-ammonia (65:35:5 v:v:v). The supernatant solution remaining after sedimentation of the total particulate fraction, containing approximately 50% of the ^{14}C added as choline, was passed through a Dowex 50 (H^+ form) column. All of the ^{14}C was retained on the column. As shown in Table I some 20% of the adsorbed ^{14}C was eluted with 2 N NH_4OH, and the remainder was eluted with 3 N HCl. Data for choline-^{14}C are included for comparison. The conclusion that the bulk of the ^{14}C not incorporated into membrane bound lecithin is unused choline-^{14}C was supported by separation of the soluble fraction by paper chromatography (solvent-1-butanol-propionic acid-water 15:9:8 v:v:v) and autoradiography. This choline remaining after incorporation into membrane lipids has ceased is apparently no longer accessible to the lecithin synthesizing mechanism; when additional choline-^{14}C was applied to the endosperm surface after 6 hr, incorporation into the particulate fraction was resumed.

Resolution of the Particulate Components. Nine ml of crude homogenate obtained from endosperm after 24 hr of exposure

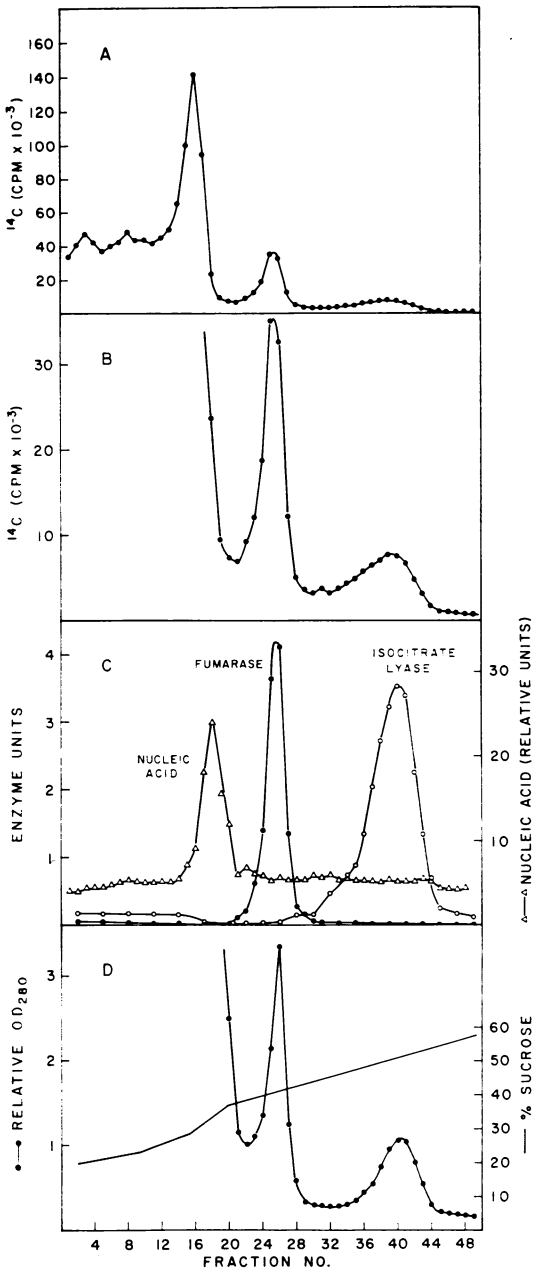

FIG. 2. Sucrose density gradient separation of particulate components of choline-^{14}C treated castor bean endosperm tissue. Ten endosperm halves were incubated for 24 hr at 30 C following application of choline-^{14}C. The tissue was homogenized and fractionated as described in the "Materials and Methods." Enzyme units: fumarase activity (●), μmoles per min per fraction (0.6 ml); isocitrate lyase activity (○), μmoles per min per fraction × 20. Nucleic acid units: percentage of nucleic acid per fraction as estimated as a function of absorbancy at 280 nm and 260 nm by the method of Warburg and Christian (22).

to choline-^{14}C were centrifuged on a 32 to 60% sucrose gradient. The data shown in Figure 2 were obtained. Three peaks of organelle or membrane-bound radioactivity were separated (Fig. 2, A and B), coinciding with visible protein bands (Fig. 2D). The distribution of isocitrate lyase across the gradient (Fig. 2C) identified the densest peak (1.24 g per cm^3) as glyoxysomes, and that of fumarase, with mean buoyant density 1.18 g per cm^3, showed that the intermediate band was mitochondrial (Fig. 2C). There was virtually no overlap of the enzyme markers between the organelles and the amounts of these enzymes in the supernatant solution were extremely small, showing that essentially no breakage of mitochondria or glyoxysomes had occurred. The bulk of the ^{14}C was recovered in the lightest band at 1.11 to 1.13 g per cm^3, which apparently includes membranes derived from endoplasmic reticulum (2, 19). Analysis for nucleic acid across the gradient (Fig. 2C) showed a single major peak with a mean density slightly higher than that of the membrane fraction. Presumably this peak represents ribosomes which are still sedimenting at the end of the 4-hr centrifugation.

Kinetics of Incorporation of Choline-^{14}C into Organelles. Choline-^{14}C was applied to a series of samples of endosperm tissue. At intervals over a 12-hr period, samples were taken for the preparation of crude homogenates. From one portion of the crude homogenates (4 ml) mitochondria and glyoxysomes were separated on a 32 to 60% sucrose gradient (Fig. 2). Another portion of the crude homogenates (4 ml) was layered on a 20 to 40% gradient (see "Materials and Methods") and centrifuged together over the 60% sucrose cushion. In this latter gradient, the light membranous fraction (tubes 11–20) is separated from soluble components and the glyoxysomes and mitochondria. The total ^{14}C content of each of the three components, *i.e.*, light membranous fraction, mitochondria, and glyoxysomes, was determined (Fig. 3).

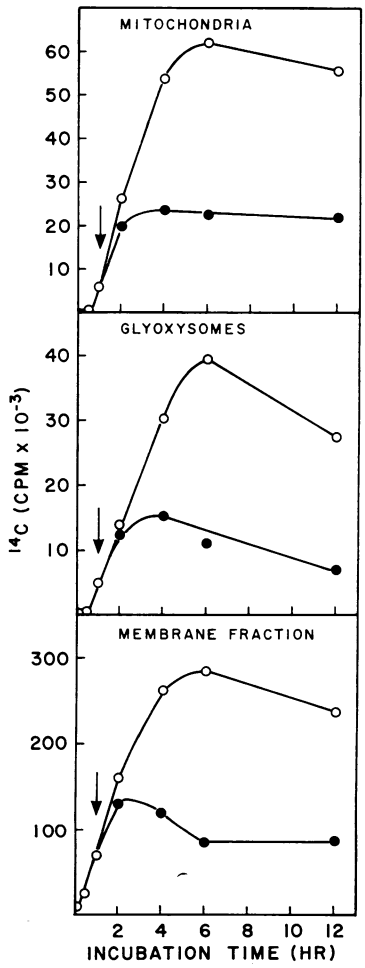

FIG. 3. Progress of ^{14}C incorporation into mitochondria, glyoxysomes, and the light membranous fraction of castor bean endosperm tissue after the application of choline-^{14}C at zero time. A 125-fold excess of unlabeled choline was applied to one set of samples after 1 hr (●); the rest received no further additions (○).

The curves with open circles show the progress of incorporation when choline-^{14}C was added at zero time and no further additions were made. During the 1st hr, the light membranous fraction became clearly labeled, whereas there was a distinct lag in the labeling of glyoxysomes and mitochondria. Incorporation into all three fractions continued for 6 hr, with the light membranous fraction the most heavily labeled throughout this time. After 6 hr when the available choline-^{14}C had been exhausted, the ^{14}C content of the glyoxysomes (roughly 10% of the total incorporated) showed a marked decline. The closed circles show the response when, as indicated by the arrows in Figure 3, a 125-fold excess of unlabeled choline was added 1 hr after the application of choline-^{14}C. Incorporation into mitochondria and glyoxysomes continued for the subsequent 3 hr, but at a diminishing rate and much more slowly than in the controls not receiving the "chase" of unlabeled choline. After 6 hr the ^{14}C content of the mitochondria declined quite slowly, while that of the glyoxysomes showed a steeper decline. The light membranous fraction showed distinctly different behavior during the chase; ^{14}C was lost rather sharply during the 2- to 6-hr period, and thereafter it remained essentially constant.

In Figure 4 the incorporation data for the first 6 hr of continuous exposure to choline-^{14}C (*i.e.*, without the chase) are expressed as percentages of total recovered in the three fractions. At the earliest times essentially all of the ^{14}C is present in the light membranous fraction, and ^{14}C appears in the mitochondria and glyoxysomes after a distinct lag (Fig. 4). Over 70% of the ^{14}C remains in the light membranous fraction at 6 hr, but during the 1- to 6-hr period ^{14}C is clearly acquired by the glyoxysomes and mitochondria while it is being lost from the light membranous fraction (Fig. 4). This reciprocal behavior is that expected if a part of the light membranous fraction subsequently gives rise to the membranes of the glyoxysomes and mitochondria.

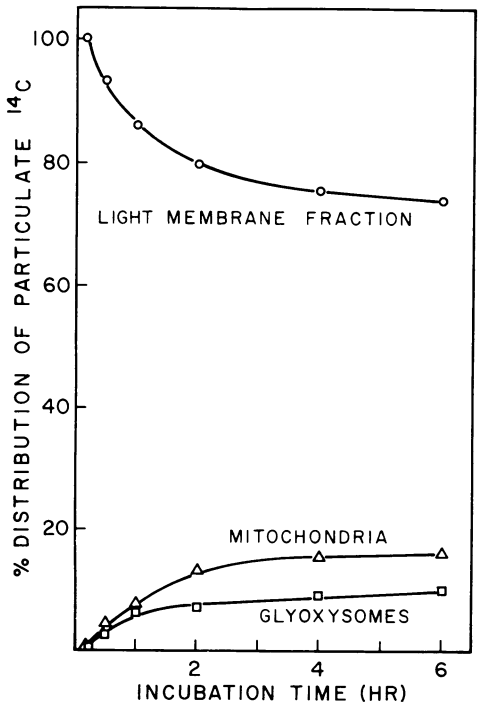

FIG. 4. Variation with time of the per cent distribution of the total particulate ^{14}C in the light membranous fraction (○), mitochondria (△), and glyoxysomes (□) from 4-day-old castor bean endosperm following the application of choline-^{14}C (calculated from the data given in Fig. 3).

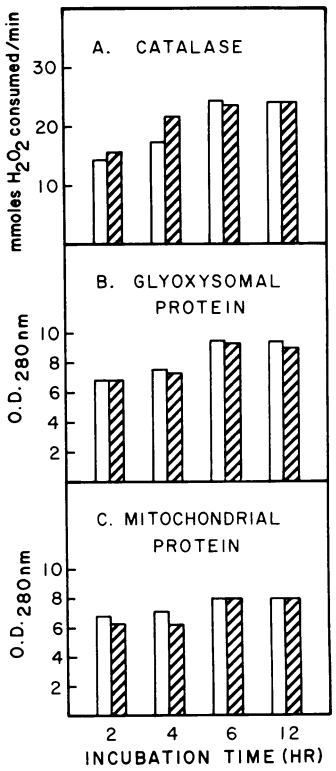

FIG. 5. Glyoxysomal catalase activity (A), glyoxysomal protein (B), and mitochondrial protein (C) recovered in the separated organelle fractions during the choline-^{14}C experiments shown in Figure 3. The closed bars represent samples which received a chase of unlabeled choline and the open bars samples which received only choline-^{14}C.

Table II. *Decline of the Lecithin-^{14}C Component of Organelles of Choline-^{14}C Labeled Castor Bean Endosperm Tissue*

Half lives were calculated from semilogarithmic plots of the data in Figure 3.

Fraction	Calculated Half-life	
	No chase	Chase of unlabeled choline
	hr	
Light membranous fraction		4
Mitochondria	48	52
Glyoxysomes	8	11

DISCUSSION

The changing rate of incorporation of choline-^{14}C into the total particulate fraction (and into its individual constituents, data not shown) parallels closely the changing total activity of catalase, a glyoxysomal marker enzyme (Fig. 1). Incorporation was most rapid when organelles were accumulating (3–5 days), but a declining rate was observed at a time when there was a net loss of both mitochondria and glyoxysomes (5–7 days). These changes appear reasonable and are not apparently due simply to differences in the sizes of lecithin precursor pools during development; from the kinetics of incorporation these pools must be small at all times.

Endosperm tissue from 4-day-old seedlings was used in the subsequent labeling experiments, undertaken to establish the half life of membrane bound lecithin in the various organelle

fractions (Fig. 3). Since separate endosperm samples were used for each time point in these experiments, it was necessary to select the replicate samples carefully from seedlings at the same developmental age and to ensure that homogenization techniques resulted in quantitative recovery of the organelle fractions.

The data presented in Figure 5 show the yields of mitochondrial and glyoxysomal protein and glyoxysomal catalase recovered in organelle fractions, separated by sucrose density gradient centrifugation, during choline-^{14}C pulse and pulse-chase experiments (from which the data in Figs. 3 and 4 were derived).

The reproducibility of homogenization is shown by the good agreement in the recovery of organelle protein and glyoxysomal catalase at comparable times during pulse and pulse-chase experiments. In intact seedlings, a net increase in organelle protein and marker enzymes (*e.g.*, catalase, Fig. 1) was still occurring between 4 and 4½ days of germination. The increase in recovered organelle protein and enzyme which occurred during incubation of excised endosperm tissue (Fig. 5) indicates that development continued normally for the duration of these experiments.

On the basis of these considerations, we conclude that the decrease in lecithin-^{14}C in recovered organelle fractions observed in pulse-chase experiments with choline-^{14}C (Fig. 3) represents turnover of this membrane constituent. These data were used to calculate the half-life of membrane-bound lecithin in the organelle fractions (Table II). Half-lives were also calculated from the declines in lecithin-^{14}C in the organelle fractions between 6 and 12 hr in the experiments in which no chase was given. In these, choline-^{14}C accessible to enzymes synthesizing lecithin apparently becomes depleted after 6 hr and incorporation stops, although the tissue is still capable of lecithin synthesis as shown by a second addition of choline-^{14}C at this time. Endogenous choline apparently acts as a chase in these experiments. Half-lives calculated from both kinds of experiment (Table II) were in good agreement. The lecithin component of mitochondria is evidently more stable than that in glyoxysomal membranes. Further data on the turnover rates of other membrane constituents and enzyme proteins are necessary to establish whether the values obtained for lecithin are a measure of organelle turnover.

The light membranous fraction apparently consists of a rapidly turning over component(s) and a more stable one(s) (Fig. 3, pulse chase experiment). Following a short (10 min) exposure to choline-^{14}C, lecithin-^{14}C was found only in the light membrane fraction. Longer exposures were required before lecithin-^{14}C was recovered in mitochondria and glyoxysomes. Incorporation into the light membrane fraction began at once and was linear with time, whereas that into mitochondria and glyoxysomes became linear after an initial lag phase (Figs. 3 and 4). These data seem best accounted for by supposing that lecithin is synthesized on intracellular components recovered in the light membrane fraction which subsequently gives rise to the membranes of other organelles. This conclusion is supported by previous studies on the intracellular distribution of particulate enzymes of lecithin biosynthesis (10, 15, 23). Phosphorylcholine-cytidyl transferase (EC 2.7.7.15) and phosphorylcholine-glyceride transferase (EC 2.7.8.2) are in fact essentially confined to components recovered in our light membrane fraction (Lord, Kagawa, and Beevers unpublished).

The reciprocal labeling pattern in Figure 4 is highly suggestive. Although the material recovered in the light membrane fraction is heterogeneous, a major component is undoubtedly derived from the endoplasmic reticulum. The association of strands of endoplasmic reticulum with microbodies seen in electron micrographs (5, 13, 20, 21) may thus have a biochemical foundation and developmental significance.

Acknowledgment—We thank Dr. W. E. Domingo of the Baker Castor Oil Company, La Mesa, California for a generous supply of castor beans.

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