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Enzymes of Phospholipid Metabolism in the Endoplasmic Reticulum of Castor Bean Endosperm[1]

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ABSTRACT

The intracellular location of several enzymes concerned with phospholipid metabolism was investigated by examining their distribution in organelles separated on sucrose gradients from total homogenates of castor bean (*Ricinus communis* var. Hale) endosperm. The enzymes phosphatidic acid phosphatase, CDP-diglyceride-inositol transferase, and phosphatidylethanolamine-L-serine phosphatidyl transferase were all primarily or exclusively confined to membranes of the endoplasmic reticulum. These results and those reported previously on lecithin synthesis establish a major role of the endoplasmic reticulum in phospholipid and membrane synthesis in plant tissues.

We have described recently the isolation from castor bean endosperm of a discrete membranous fraction which was shown to be derived from the endoplasmic reticulum (12, 16). This material, recovered at an equilibrium density of 1.12 g/cm^3 when the whole homogenate was centrifuged on a sucrose gradient, proved to be the exclusive intracellular site of the enzyme producing lecithin, phosphorylcholine-glyceride transferase (15). Since this fraction was clearly separated from other organelle fractions and also from other components which are present in crude microsomal preparations, it seemed appropriate to examine it further for other enzymes of phospholipid metabolism.

Microsomal preparations (usually that material sedimenting at 10,000–100,000*g*) from animal tissues have been shown to contain enzymes concerned with the synthesis of lecithin, phosphatidylserine (by exchange), phosphatidylethanolamine (from CDP-ethanolamine), and phosphatidylinositol (2, 4, 8, 18, 22). Phosphatidic acid phosphatase was found predominantly in a fraction sedimenting between the mitochondrial and microsomal fractions in differential centrifugation and was thus thought to be lysosomal (26, 32). From previous studies with plant systems, it had been concluded that lecithin synthesis occurred in microsomal (3, 11) or Golgi fractions (19, 21), that phosphatidylserine synthesis was microsomal (30), and that mitochondria were the site of phosphatidylinositol synthesis (28). We have shown previously that the presence of "microsomal" enzymes in low speed fractions obtained by differential centrifugation is due to contamination by microsomes and that a much clearer picture is obtained when the membranes of the endoplasmic reticulum are separated directly from the homogenate on sucrose gradients (15). The observations reported here establish clearly that several other enzymes of phospholipid metabolism are associated with this fraction.

MATERIALS AND METHODS

Seeds of castor bean (*Ricinus communis* var. Hale) were soaked in running tap water for 1 day and germinated in moist vermiculite in darkness at 30 C.

Homogenization. Twenty endosperm halves, removed from 4-day-old seedlings, were chopped for 15 min with a single razor blade in 8 ml of grinding medium contained in a Petri dish on ice. The grinding medium contained 150 mM Tricine buffer pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA (pH 7.5), and 13% (w/w) sucrose.

The crude homogenate was filtered through two layers of nylon cloth, and the volume of the filtered homogenate was adjusted to 10 ml with grinding medium. Cell debris was removed by centrifuging at 270*g* for 10 min; the supernatant solution was taken as the crude homogenate.

Fractionation of Cellular Components by Sucrose Density Gradient Centrifugation. Five ml of the crude homogenate were layered on gradients composed of (*a*) a 6-ml cushion of 60% (w/w) sucrose, (*b*) 20 ml of sucrose solution increasing linearly in concentration from 32 to 60% (w/w) sucrose, and (*c*) a 5-ml layer of 20% (w/w) sucrose, contained in a 37.5-ml tube. All sucrose solutions were prepared in 1 mM EDTA (pH 7.5).

The gradients were centrifuged at 20,000 rpm for 4 hr in a Beckman Model L2-65B centrifuge equipped with a Spinco Model SW 27 rotor and maintained at 0 C. Separation into serial 0.6-ml fractions and recording of the A_{280} was accomplished with an Isco fractionator. Sucrose content of the individual samples was determined refractometrically.

Enzyme assays. Fumarase (23), malate synthetase (9), and cytochrome *c* reductase (27) were assayed as described. Phosphatidic acid phosphatase was assayed by a modification of the method of McCaman *et al* (17). The reaction mixture consisted of 4 mM phosphatidic acid (Schwarz/Mann) and 100 mM tris-HCl (pH 7.4) in a final volume of 100 µl. The reaction was started by addition of enzyme, and the samples were incubated at 30 C for 1 hr. Blanks without substrate were used for each gradient fraction. The reaction was stopped by the addition of 100 µl of 10% trichloroacetic acid. After chilling and centrifuging, the soluble phosphate released from the

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substrate was assayed by adding 20 μl of the supernatant to 1.0 ml of reagent prepared fresh by dissolving 3 g of ascorbic acid in 120 ml of water and adding 3 ml each of 10 N H_2SO_4 and 5% ammonium molybdate. After incubating the samples for 20 min at 38 C, absorbance was determined at 820 nm.

The sensitivity of this assay makes it imperative that extremely clean glassware be used. Our glassware was soaked sequentially in 1 N KOH, distilled water, 6 N HCl, and rinsed in several changes of distilled water.

CDP-diglyceride-inositol transferase was assayed (28) in a final volume of 500 μl containing 50 mM tris-HCl (pH 8.0), 2 mM CTP, 2 mM $MnCl_2$, and 0.1 μc of *myo*-UL-^{14}C-inositol (0.47 μmole). The mixture was incubated at 30 C for 60 min, and the reaction was terminated by the addition of 2.0 ml of absolute ethanol. Two ml of chloroform were then added, and the mixture was extracted twice with 5 ml of 2 M KCl and twice with distilled water to remove unreacted inositol. The chloroform phase was transferred to scintillation vials, evaporated to dryness, 10 ml of scintillation fluid (100 g of naphthalene, 6 g of PPO, in 1 liter of dioxane) were added, and ^{14}C was assayed in a Beckman LS-200B scintillation counter.

Phosphatidylethanolamine-L-serine Phosphatidyl Transferase. The reaction (adapted from 24) was carried out for 30 min at 30 C in a total volume of 500 μl containing 40 mM HEPES buffer (pH 7.8), 8 mM $CaCl_2$, and 1 $\times$ 10^5 dpm DL-serine-3-^{14}C (15.5 nmoles). The reaction was started by adding the serine and stopped by adding 2.0 ml of ethanol. Extraction,

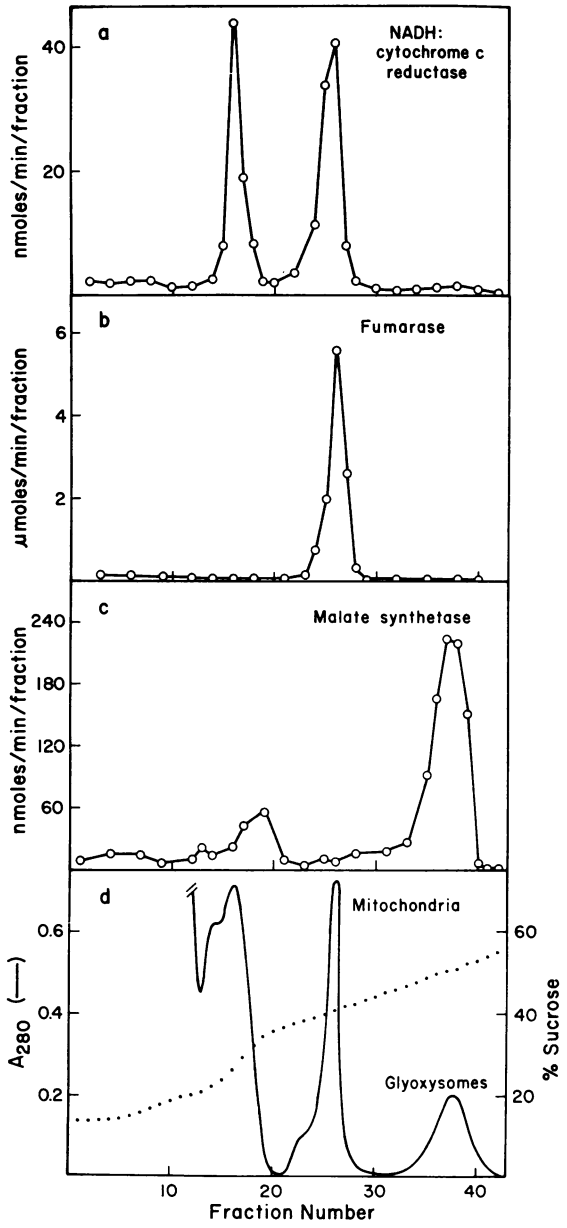

FIG. 1. Localization of endoplasmic reticulum, mitochondria, and glyoxysomes in sucrose gradients using the marker enzymes NADH-cytochrome *c* reductase (a), fumarase (b), and malate synthetase (c). Absorbance at 280 nm (protein) as a continuous trace and sucrose concentration of individual fractions are presented in (d).

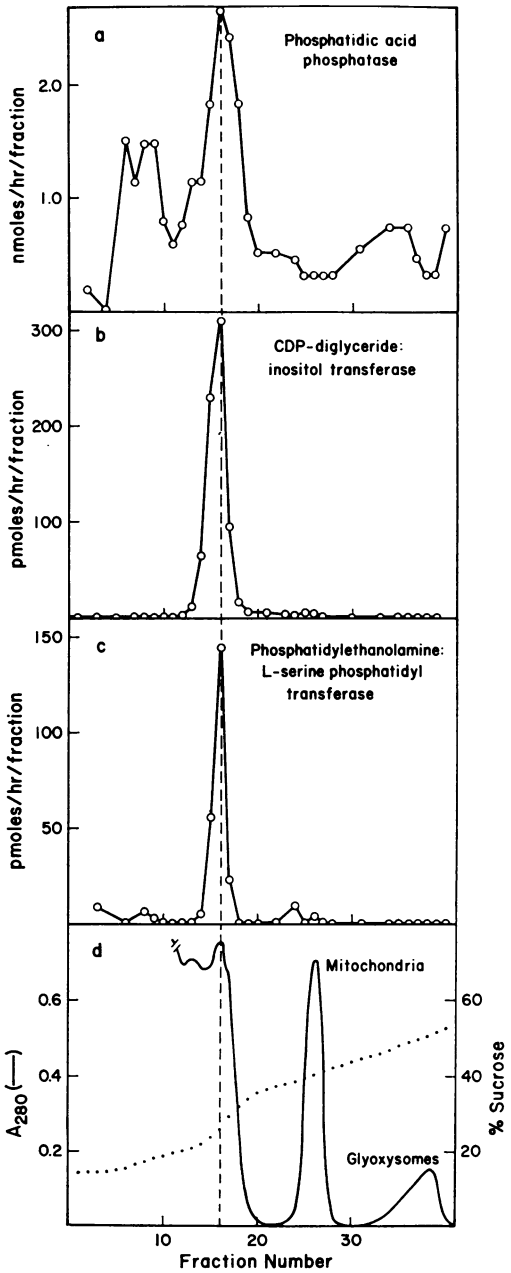

FIG. 2. Distribution of (a) phosphatidic acid phosphatase; (b) CDP-diglyceride: inositol transferase; and (c) phosphatidyl-ethanolamine: L-serine phosphatidyl transferase in the sucrose gradient. In (d) the continuous protein trace (—) and the sucrose concentration in sequential samples (···) from the gradient are shown.

Table I. *Requirements for the Synthesis of Phosphatidylinositol*

The complete system in a final volume of 500 μl contained: 50 mM tris-HCl (pH 8.0), 2 mM $MnCl_2$, and 0.1 μc of *myo*-UL-^{14}C-inositol (0.47 μmole).

Assay system	Phosphatidylinositol produced	
	cpm/hr	*pmoles/hr*
Complete	18,800	39.5
− Enzyme	40	0.08
− $MnCl_2$	200	0.4
Complete: boiled enzyme	70	0.15

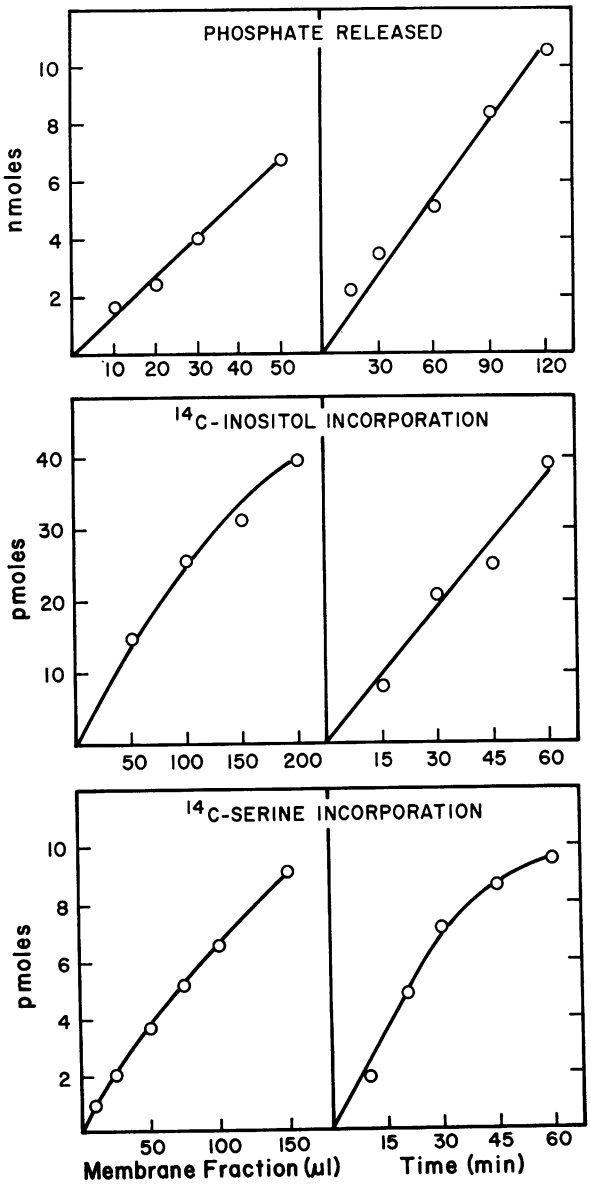

FIG. 3. Effects of increasing amounts of protein from the endoplasmic reticulum fraction (left) and incubation time (right) on the enzyme reactions. In the upper curves the amounts of phosphate released from phosphatidic acid are shown. Incorporation of ^{14}C-inositol and ^{14}C-serine into chloroform-soluble material are shown in the center and lower pairs of curves. Reaction conditions are described in "Materials and Methods." The protein content of 100 μl membrane fraction was approx 120 μg.

washing, and ^{14}C assay were carried out as described above for CDP-diglyceride-inositol transferase.

The chloroform-soluble products of the radioisotopic assays appeared as single spots after thin layer chromatography (5) and autoradiography.

RESULTS

The data presented in Figure 1 show the distribution of protein (d) and the locations of the various major organelle fractions in the gradients described. Fumarase activity locates the mitochondrial fraction at 1.18 g/cm^3 (Fig. 1b). NADH-cytochrome *c* reductase (Fig. 1a) shows a double peak. The activity in the mitochondrial fraction at density 1.18 is sensitive to antimycin, and that at density 1.12 g/cm^3 is resistant and marks the endoplasmic reticulum (16). The glyoxysomes were located at 1.24 g/cm^3 by the assay for malate synthetase (Fig. 1c).

Assays for phosphatidic acid phosphatase (Fig. 2a) across a similar gradient gave a major peak corresponding to the endoplasmic reticulum fraction (1.12 g/cm^3). No activity was specifically associated with the mitochondrial or glyoxysomal fractions, although some soluble enzyme was present higher in the gradient. No activity was observed when the endoplasmic reticulum fraction was boiled prior to assay. Neither glucose 6-phosphate nor *o*-carboxyphenylphosphate was hydrolyzed by this fraction.

Synthesis of phosphatidylinositol was found exclusively in the 1.12 g/cm^3 fraction (Fig. 2b) and exhibited a profile similar to that for lecithin synthesis (15). Enzyme activity was obtained without adding the second substrate, CDP-diglyceride, suggesting that an endogenous source of this compound is present. The fact that a 2.5-fold stimulation of activity was obtained when CDP-diglyceride was added indicates strongly that direct synthesis, rather than exchange (8), was occurring. A similar stimulation by CDP-diglyceride was also observed in the mitochondrial fraction, but the activity in this fraction was only about 4% of the total and is therefore probably due to microsomal contamination. These data indicate that the lack of significant activity in the mitochondrial fractions of the gradient is not due to lack of the second substrate. Further characterization of the requirements for phosphatidylinositol synthesis showed (Table I) that, as found previously by Sumida and Mudd (28), Mn^{2+} is required for the

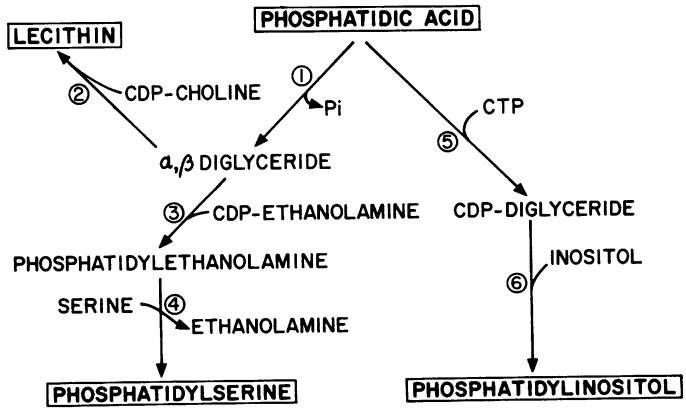

FIG. 4. Pathways of synthesis of three major phospholipids (after Kennedy, 14). Enzymes catalyzing reactions 2, 4, and 6 producing, respectively, lecithin, 2, phosphatidylserine, 4, and phosphatidylinositol, 6, as well as phosphatidic acid phosphatase (reaction 1) have now been shown to occur in the membranes of the endoplasmic reticulum from castor bean endosperm.

reaction to occur. However, in contrast to the situation in their cauliflower system, the addition of CTP (or CDP) had no effect on the synthesis of phosphatidylinositol in the castor bean fraction, suggesting that CDP-diglyceride is not being formed under the conditions of the assay.

Assay of the activity of phosphatidylethanolamine-L-serine phosphatidyl transferase across the gradients also showed a sharp peak in the endoplasmic reticulum membrane fraction with only trace activity in the mitochondrial fraction and none elsewhere (Fig. 2, c).

As shown in Figure 3, the reaction rate for each of the three enzymes was proportional to enzyme concentration and proceeded linearly with time for at least 30 min. Assays of gradient fractions were carried out within the limits of time and enzyme concentration shown in this figure.

DISCUSSION

In Figure 4, a schematic pathway of the synthesis of commonly occurring phospholipids is shown (14). In this and the preceding papers of this series (12, 15, 16), evidence has been presented for the exclusive or primary localization of reactions 1, 2, 4, and 6 in the membranes of the endoplasmic reticulum from the endosperm of young castor bean seedlings.

It is therefore clear that, at least in this tissue, these membranes are the site of synthesis of at least three phospholipids, phosphatidylcholine (lecithin), phosphatidylinositol, and phosphatidylserine which are known to be major components of membranes of cellular organelles from a variety of animal (18) and plant (13) tissues and specifically of microsomal, mitochondrial, and glyoxysomal membranes of castor bean endosperm as shown recently by Donaldson *et al.* (5).

These findings, of interest in themselves, re-emphasize the problem of transferring these phospholipids from the site of synthesis in the endoplasmic reticulum to the membranes of other organelles. Their size and relative insolubility in aqueous solutions would seem to argue against free diffusion through the cytoplasm. Two possibilities of transport can be recognized; direct incorporation of the endoplasmic reticulum membranes through continuities with the developing organelles (microbodies, 7, 10, 31; mitochondria, 1, 6, 20, 25) or transfer of individual phospholipids by special transport proteins (29). The specific role of these two mechanisms in the synthesis of a given membrane has yet to be elucidated; the castor bean endosperm system, in which rapid organelle synthesis occurs during germination, offers the possibility of a direct investigation of this problem.

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