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Intracellular Distribution of Enzymes of the Cytidine Diphosphate Choline Pathway in Castor Bean Endosperm

(lecithin/membranes/glyoxysomes/mitochondria/endoplasmic reticulum)

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ABSTRACT **The occurrence and subcellular distribution of enzymes of the cytidine diphosphate choline pathway of lecithin synthesis have been examined. Choline kinase (EC 2.7.1.32) was completely soluble, while phosphorylcholine-cytidyl transferase (EC 2.7.7.15) and phosphorylcholine-glyceride transferase (EC 2.7.8.2) were associated with particulate fractions. Although components sedimenting at 10,000 to 100,000 × *g* contained both enzymes, phosphorylcholine-cytidyl transferase and particularly phosphorylcholine-glyceride transferase were present in the 10,000 × *g* pellet, which contained the major organelles, mitochondria, and glyoxysomes. When the crude homogenate was centrifuged on a sucrose density gradient, four major bands of particulate protein were recovered. A band at density 1.24 g/cm³ contained the glyoxysomes and was devoid of phosphorylcholine-cytidyl transferase and phosphorylcholine-glyceride transferase activity. Enzyme activity was barely detectable in the mitochondria, at density 1.18 g/cm³. Phosphorylcholine-glyceride transferase was found almost exclusively in a sharp band at density 1.12 g/cm³, and phosphorylcholine-cytidyl transferase was found in the uppermost band at density 1.08 g/cm³. Thus, for the synthesis of lecithin in their membranes, the glyoxysomes and mitochondria depend on enzymes elsewhere in the cell; the final two steps in lecithin formation occur, apparently exclusively, in separate particulate cell components.**

Recent studies have shown that [^{14}C]choline is an effective precursor of membrane-bound lecithin in endosperm tissue of germinating seedlings of castor bean (1). Labeled membrane components were separated after application of [^{14}C]choline by centrifugation of homogenates on linear 30–60% (w/w) sucrose density gradients. Three particulate bands were separated on such gradients: a membranous fraction at a buoyant density of 1.12–1.13 g/cm³, mitochondria (1.18 g/cm³), and glyoxysomes (1.24 g/cm³).

The time course of [^{14}C]choline incorporation into these cellular components showed that the membranous fraction acquired [^{14}C]lecithin before the mitochondria and glyoxysomes. Apparently, the final stage of lecithin biosynthesis occurred in components of the membranous fraction, and the preformed phospholipid served as a precursor for the formation of mitochondrial and glyoxysomal membranes. Accordingly, we have investigated the distribution of enzymes involved in lecithin synthesis among cell fractions separated from castor bean endosperm.

A major pathway for the synthesis of lecithin from choline has been described by Kennedy (2). The sequence involves three enzyme-catalyzed steps: (*a*) the ATP-dependent phosphorylation of choline to phosphorylcholine catalyzed by choline kinase (EC 2.7.1.32), (*b*) the formation of CDP-choline from CTP and phosphorylcholine catalyzed by phosphorylcholine-cytidyl transferase (EC 2.7.7.15), and (*c*) the reaction between CDP-choline and diglyceride to form lecithin, catalyzed by phosphorylcholine-glyceride transferase (EC 2.7.8.2).

The three enzymes of the CDP-choline pathway have been reported from plant sources (3–5), and the importance of the pathway in relation to the synthesis of membranous cell constituents has been discussed (4, 5).

In this paper, we report the compartmentation of PChol-cytidyl transferase and PChol-glyceride transferase in two distinct components of the light membrane fraction, and we discuss their role in the biogenesis of glyoxysomes and mitochondria.

MATERIALS AND METHODS

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

Homogenization. 20 Endosperm halves, removed from 4-day-old seedlings, were homogenized by chopping for 15 min with a single razor blade in 6 ml of grinding medium contained in a petri dish on ice. The grinding medium contained 0.15 M Tricine (pH 7.5), 10 mM KCl, 10 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM EDTA (pH 7.5) and sucrose. The concentration of sucrose in this medium was 0.63 M (20% w/w) for preparation of extracts for differential centrifugation and 0.5 M (16% w/w) for extracts used in sucrose density gradient experiments. The crude homogenate was filtered through two layers of nylon cloth, and the residue was extracted with a further 4 ml of grinding medium. The volume of the filtered homogenate was adjusted to 10 ml with grinding medium, and cell debris was removed by centrifugation at 270 × *g* for 10 min. The total particulate fraction was obtained by centrifugation of the supernatant solution at 150,000 × *g* for 1 hr.

Differential Centrifugation. The 270 × *g* supernatant was successively centrifuged at 10,000 × *g* for 15 min, 40,000 × *g* for 15 min, and finally at 150,000 × *g* for 1 hr. The pellets recovered at each stage were resuspended in 1 ml of grinding medium.

Sucrose Density Gradient Centrifugation. 4 ml of the 270 × *g* supernatant was layered onto a sucrose density gradient. The gradient consisted of 20 ml of sucrose solution increasing linearly in concentration from 32 to 60% (w/w) over a 2-ml cushion of 60% (w/w) sucrose, and topped with a 5-ml layer of 20% (w/w) sucrose. Gradients were contained in 38.5 ml of

Abbreviations: PChol-cytidyl transferase, phosphorylcholine-cytidyl transferase (EC 2.7.7.15); PChol-glyceride transferase, phosphorylcholine-glyceride transferase (EC 2.7.8.2).

polyallomer tubes. All sucrose solutions were prepared in 1.0 mM EDTA (pH 7.5). Gradients were centrifuged for 4 hr at 20,000 rpm in a SW 27 rotor in a Beckman L2-65B ultracentrifuge. After centrifugation, 0.6-ml fractions were collected with an ISCO density gradient fractionator, model 640.

Enzyme Assays. Choline kinase was assayed by the method of Tanaka *et al.* (3). The reaction mixture contained, in a final volume of 0.2 ml, 20 μmol of potassium phosphate (pH 8.0), 1.5 μmol ATP, 1.5 μmol $MgCl_2$, 0.3 μmol [*methyl*-^{14}C]-choline (1.66 Ci/mol, New England Nuclear Corp.), and enzyme. The mixture was incubated at 30° for 1 hr on a shaken water bath, and the reaction was stopped by heating for 3 min on a boiling-water bath. [^{14}C]Phosphorylcholine formed during the reaction was separated by paper chromatography, and its radioactivity was determined (3). Boiled-enzyme controls showed no activity.

Phosphorylcholine-cytidyl transferase was assayed by the method of Borkenhagen and Kennedy (6). The reaction mixture contained, in a final volume of 0.5 ml, 50 μmol Tris·HCl (pH 7.0), 10 μmol $MgCl_2$, 1 μmol CTP, 0.25 μCi [*methyl*-^{14}C]phosphorylcholine (44 Ci/mol; New England Nuclear Corp.), and enzyme. The mixture was incubated at 30° for 1 hr in a shaken water bath, and the reaction was stopped by addition of 5 ml of 5% (w/v) trichloroacetic acid.

The precipitate was removed by centrifugation. 2 ml of an aqueous suspension of Norit A charcoal (10 mg/ml) was added to the supernatant to bind the [^{14}C]CDP-choline formed during the reaction. The suspension was mixed with occasional shaking for 10 min, and the charcoal was recovered by centrifugation and washed three times with 5-ml portions of water. Heating of the charcoal in 5 ml of 3 N HCl on a boiling-water bath for 30 min with occasional shaking yielded free [^{14}C]choline. The charcoal, recovered by centrifugation, was washed three times with 2-ml portions of water. The hydrolysis and washings removed all detectable radioactivity from the charcoal. The combined acid hydrolysate and washings were transferred to scintillation vials and evaporated to dryness. 10 ml of scintillation fluid was added, and the ^{14}C content was assayed in a Beckman LS-200B liquid scintillation spectrometer.

Phosphorylcholine-glyceride transferase was assayed by the method of Weiss *et al.* (7). The reaction mixture contained, in a final volume of 0.5 ml, 50 μmol of Tris·HCl (pH 7.0), 10 μmol $MgCl_2$, 5 μmol 1,2-dipalmitin (added as an emulsion in 50 μl of 0.3% (w/v) Tween 20), 0.1 μCi [*methyl*-^{14}C]CDP choline (40 Ci/mol; New England Nuclear Corp.), and enzyme. The mixture was incubated at 30° for 1 hr in a shaken water bath, and the reaction was stopped by addition of 2 ml of absolute ethanol. The precipitated protein was removed by centrifugation, and the pellet was extracted with 2 ml of ethanol. After centrifugation, the ethanol phases were combined and mixed with 3 ml of chloroform. The organic phase was washed twice with 5-ml portions of 2 M KCl, and twice with 5-ml portions of water. The residual chloroform phase was transferred to scintillation vials, evaporated to dryness, and assayed for ^{14}C.

Other Methods. Protein was determined by the method of Lowry *et al.* (8), with the use of a calibration curve prepared for crystalline bovine-serum albumin. Sucrose concentrations were determined refractometrically.

TABLE 1. *Distribution of enzymes of the CDP-choline pathway between the soluble and particulate cell fractions of endosperm from 4-day-old castor beans*

Enzyme	Total activity (nmol/hr per 20 endosperm halves)	
	Soluble	Particulate
Choline kinase	880 (99.2)*	6 (0.8)
PChol-cytidyl transferase	15 (46.6)	17.2 (53.4)
PChol-glyceride transferase	0.07 (0.4)	21 (99.6)

* Figures in parentheses show percentage of total activity.

RESULTS

The three enzymes of the CDP-choline pathway of lecithin biosynthesis are present in extracts of endosperm tissue from 4-day-old castor beans. The total activity of these enzymes extracted from 20 endosperm halves (6 g fresh weight) is shown in Table 1. Measured enzyme activities were more than adequate to account for the rate of [^{14}C]choline incorporation into membrane-bound lecithin observed *in vivo* (1). The distribution of the enzyme between the particulate (150,000 × *g* pellet) and soluble (150,000 × *g* supernatant) fractions of endosperm extracts (Table 1) is similar to that observed in experiments with animal (9, 10) and plant (4, 5) tissues. Choline kinase, which is not particulate, was not investgated further in this study.

The distribution of particulate CDP-choline pathway enzymes among fractions obtained by differential centrifugation is shown in Table 2. PChol-cytidyl transferase was present in all fractions, with the 40,000 × *g* and 150,000 × *g* pellets (the microsomal fractions) containing the greatest proportion of the particulate enzyme at the highest specific activity. PChol-glyceride transferase, also present in all particulate fractions, was recovered primarily in the 10,000 × *g* pellet (Table 2). These distribution patterns are similar to those previously reported with the same technique (4, 5, 9, 10).

However, such fractionation techniques provide only an enrichment, rather than a separation, of particulate cell components. In order to assay specific organelle fractions for associated enzyme activity, we further separated particulate fractions of castor bean endosperm by sucrose density gradient centrifugation.

4 ml of crude homogenate was applied to a gradient and, after 4 hr of centrifugation, four separate bands were visible

TABLE 2. *Distribution of particulate enzymes of the CDP-choline pathway among fractions obtained by differential centrifugation*

Fraction	PChol-cytidyl transferase (nmol/hr per)		PChol-glyceride transferase (nmol/hr per)	
	20 Endosperm halves	mg of protein	20 Halves	mg of protein
10,000 × *g* pellet	5.46	0.38	12.35	1.62
40,000 × *g* pellet	3.60	0.67	5.50	1.53
150,000 × *g* pellet	8.22	0.98	3.12	0.99
150,000 × *g* supernatant	15.06	0.17	0.07	0.001

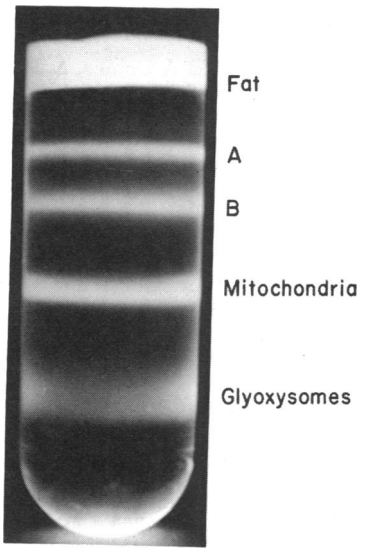

FIG. 1. Results of sucrose density gradient centrifugation.

in the gradient (Fig. 1). Mitochondria were recovered at a mean sucrose density of 1.18 g/cm³ and glyoxysomes at a density of 1.24 g/cm³. The interposition of 5 ml of 20% sucrose between the gradient and sample (see *Methods*) allowed separation of the light membrane fraction (1) into two distinct bands, *A* and *B*, at densities of 1.08 g/cm³ and 1.12 g/cm³. Prolonged (24 hr) centrifugation of gradients showed that band *B*, mitochondria, and glyoxysomes had reached their equilibrium buoyant density after 4 hr, but band *A* continued to move into the gradient.

Phosphorylcholine-cytidyl transferase

The distribution of PChol-cytidyl transferase among gradient fractions after 4 hr of centrifugation showed this enzyme to be associated primarily with particulate components of band *A* (Fig. 2). Some activity was present in band *B*, and, to a very much lesser extent, in mitochondria. No enzyme activity was found in glyoxysomes. No soluble PChol-cytidyl transferase activity remained at the top of the gradient, whereas a considerable amount remained in the supernatant solution after differential centrifugation (Tables 1 and 2). In those experiments, the grinding medium contained 20% (w/w) sucrose (density 1.08 g/cm³), rather than the 16% (w/w) sucrose (density 1.06 g/cm³) used when extracts were prepared for gradient centrifugation. The density of the grinding medium and the short duration of differential centrifugation apparently prevented the complete sedimentation of particulate components containing the enzyme.

Phosphorylcholine-glyceride transferase

After gradient centrifugation, this enzyme was recovered in band *B*. Activity was not detected in band *A* or in glyoxysomes. Mitochondria contained extremely low, but detectable, PChol-glyceride transferase activity. The presence of considerable PChol-glyceride transferase activity in the 10,000 × *g* pellet obtained by differential centrifugation (Table 2), which contained the mitochondria and glyoxysomes, was thus apparently due to contamination by particulate material that is recovered almost exclusively in band *B* after gradient centrifugation.

The distribution of PChol-glyceride transferase across the

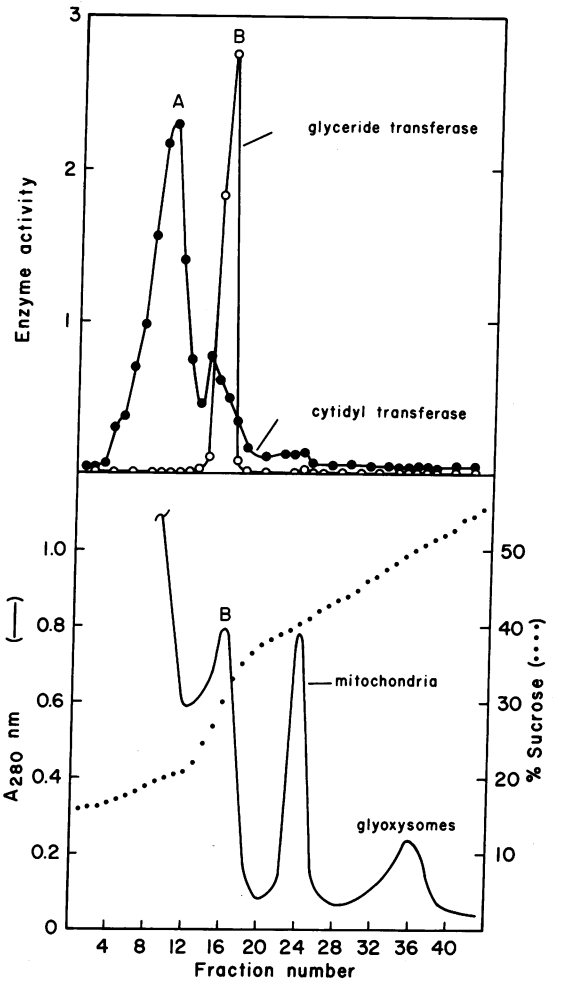

FIG. 2. Distribution of enzymes among fractions of the gradient shown in Fig. 1. Enzyme units: nmole of product formed per hr per gradient fraction.

gradient shown in Fig. 2 was obtained by assay of the enzyme in the presence of added diglyceride emulsified in Tween-20. However, further examination of these assay conditions showed that the activity of the enzyme present in material recovered from band *B* on the gradient was not significantly enhanced by additional diglyceride, but was severaly inhibited by Tween (Table 3). The product of the enzyme reaction in the absence of exogenous diglyceride and Tween-20 chromatographed as a single radioactive component; it was identified as lecithin.

TABLE 3. *Effects of added diglyceride and Tween-20 on PChol-glyceride transferase activity*

Assay	Radioactivity in lecithin (cpm)
Complete	20,629
Complete; boiled enzyme	205
Minus diglyceride	17,072
Minus diglyceride, minus Tween	48,061

The complete reaction mixture contained, in a final volume of 0.5 ml, 50 μmol of Tris·HCl (pH 7.0), 10 μmol of $MgCl_2$, 4 μmol of 1,2-dipalmitin; 120 μg of Tween-20, 0.1 μCi of [*methyl*-^{14}C]CDP-choline (40 Ci/mol), and 100 μl of enzyme obtained from band *B* after sucrose density gradient centrifugation.

When the enzyme was assayed across the gradient by omitting diglyceride and Tween-20 from the reaction, activity was again confined to band *B* (data not shown).

DISCUSSION

Studies on the origin of the membranes of rat-liver mitochondria have suggested that newly synthesized lecithin is transferred *in vivo* from the endoplasmic reticulum to mitochondria (11, 12). It has been reported that isolated rat-liver mitochondria can synthesize lecithin via the CDP-choline pathway (13–15), but this is probably due to contamination of mitochondrial preparations (9, 16). The general role of the endoplasmic reticulum in lecithin biosynthesis in animal (9) and plant (4.5) tissue is supported by the demonstration that the enzymes PChol-cytidyl transferase and PChol-glyceride transferase are found together in cell components sedimenting between 10,000 × *g* and 100,000 × *g* ("microsomes"). These observations, from fractions obtained by differential centrifugation, suggest that these two particulate enzymes involved in lecithin biosynthesis are present in the same cytoplasmic structure.

The sucrose gradients used in the present work with castor bean endosperm enabled us to separate cleanly mitochondria and glyoxysomes from "microsomal" components, and further separated the "microsomal" fraction into two well-defined bands (bands *A* and *B*, Fig. 1). The distribution of particulate enzymes of the CDP-choline pathway across the gradient established that PChol-cytidyl transferase is primarily associated with components present in band *A*, while PChol-glyceride transferase is confined to components of band *B* (Fig. 2).

The absence of enzymes of the CDP-choline pathway from separated glyoxysomes, and their presence in extremely low amounts in mitochondria (Fig. 2), show that lecithin, an important membrane constituent of these organelles (1), is not synthesized by the organelles themselves.

Electron microscopic examination of microbodies from various tissues has frequently shown a close association between these organelles and sections of the endoplasmic reticulum (17, 18); it has been suggested that microbodies arise from the endoplasmic reticulum (for a review, see ref. 18). A similar association between the outer membrane of mitochondria and the endoplasmic reticulum has also been reported (19–22). The demonstration that PChol-glyceride transferase was confined to band *B* after sucrose density gradient centrifugation (Fig. 2) indicates that this is the fraction responsible for the formation of the lecithin ultimately found in other organelles of the endosperm tissue. Evidence will be presented elsewhere to show that this fraction originates from the endoplasmic reticulum. One important consideration is that the distribution of NADPH-cytochrome *c* reductase (EC 1.6.2.3) (23, 24) across the gradient coincides exactly with that of PChol-glyceride transferase.

The synthesis of CDP-choline, one of the precursors of lecithin, occurs at a different cellular site, as shown by the confinement of PChol-cytidyl transferase to band *A* in the sucrose gradients. The identity of this component is not known.

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