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Biosynthesis of Cytidine 5′-Diphosphate-diacylglycerol in Endoplasmic Reticulum and Mitochondria of Castor Bean Endosperm[1]

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ABSTRACT

Cytidine 5′-triphosphate (CTP):phosphatidate cytidyltransferase from the endoplasmic reticulum and mitochondria of *Ricinus communis* L. var Hale was characterized. The endoplasmic reticulum enzyme has a pH optimum of 6.5 and a divalent cation is required, Mn^{2+} being preferred and giving maximum activity at 2.5 millimolar. The estimated K_m for CTP is 16.7 micromolar, but that for phosphatidate could not be determined accurately. The activity was inhibited by both deoxycholate and Triton X-100 at concentrations as low as 0.01% (w/w).

The mitochondrial enzyme has a pH optimum of 6.0 and a divalent cation requirement similar to that of the endoplasmic reticulum. Maximum stimulation of the reaction by substrates occurred with 1.5 millimolar phosphatidate (from egg phosphatidylcholine) and about 400 micromolar CTP. The apparent K_m for phosphatidate could not be estimated accurately since activity was obtained in the absence of added lipid, apparently utilizing endogenous substrate. The K_m estimated for CTP was altered by the presence of the detergent Triton X-100; in its absence the value was 33.3 micromolar, but in its presence the value was 66.7 micromolar. Inclusion of 0.6% (w/w) Triton X-100 in the assay mixture stimulated the activity about 2.5-fold.

CDP-diacylglycerol is a central intermediate in the synthesis of phospholipids, serving as a precursor for phosphatidylglycerol (and thereby cardiolipin), phosphatidylinositol and, in some organisms, phosphatidylserine (6-8,10, 12, 15). It is a branch-point product which exists at very low concentrations within the membranes (7, 8, 10, 12, 15). The enzyme involved in this synthesis may, therefore, play a role in regulation of phospholipid levels, and an understanding of the characteristics of the enzyme is critical toward understanding its role in phospholipid synthesis.

The enzyme which catalyzes the reaction is CTP:Phosphatidate cytidyltransferase (EC 2.7.7.41). The activity in plants has been reported from mitochondria and microsomes of cauliflower inflorescences (1, 3, 4, 19, 20) as well as the inner mitochondrial membranes of mung bean hypocotyls (5) and castor bean endosperm (17). This report describes the characteristics of the microsomal and mitochondrial enzymes from castor bean endosperm. Comparisons of those characteristics are made with the enzyme from other sources.

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MATERIALS AND METHODS

Materials. CDP-diacylglycerol, phosphatidic acid, and lysophosphatidic acid (all derived from egg lipids), phosphatidylinositol (yeast), and synthetic didecanoyl and dioleoyl phosphatidic acids were from Serdary Research Laboratories, Inc. (London, Ontario, Canada). Triton X-100, sodium deoxycholate, all buffers, and nonradioactive CTP were from Sigma Chemical Company. The salts were from J. T. Baker Co. and the sucrose from Mallinkrodt. [^{3}H]Cytidine 5′-triphosphate was purchased from ICN.

Germination Conditions. Castor bean seeds (*Ricinus communis* L. var Hale) were soaked overnight in running tap water, planted in moist vermiculite, and germinated 4 to 5 d in the dark at 30°C in a humidified growth chamber (90% RH).

Homogenization and Cell Fractionation. These procedures were similar to those previously described (14). Thirty endosperm halves were chopped in 11 ml of homogenization medium, contained in a Petri dish on ice, for 15 min with a single razor blade. The homogenization medium consisted of 150 mM Tricine buffer (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA (pH 7.5), and 17% (w/w) sucrose (14). Organelle separation was performed utilizing isopycnic sucrose density gradients which contained 3 mM EDTA in addition to the sucrose (14). The organelle fractions were assayed directly from the gradients.

Assays. The microsomal CTP:phosphatidate cytidyltransferase was assayed at 37°C for 30 min in a final volume of 0.5 ml. The assay solution contained 10 mM Mes buffer (pH 6.5), 7.5 mM $MnCl_2$, 2.5 mM phosphatidate, and 120 μM [^{3}H]-CTP (32.2 mCi/mmol). The reaction was initiated by the addition of an aliquot of the gradient microsomal fraction, usually containing 60 to 100 μg of protein.

The mitochondrial enzyme was assayed under the same conditions except that the assay mixture normally contained 10 mM Mes (pH 6.0), 2.5 Mm $MnCl_2$, 0.06% (w/w) Triton X-100, 1.5 mM phosphatidate (egg), and 300 μM CTP (32.2 or 18.2 mCi/mmol). The reaction was initiated by the addition of 70 to 110 μg of mitochondrial fraction protein.

The reactions were stopped by the addition of 3.3 ml of chloroform-methanol-water (1:2:0.3, v/v) and the lipid products were extracted by the method of Bligh and Dyer (2). To determine the radioactivity in the lipid fraction, the chloroform fraction was dried in a Biovial (Beckman Instrument Co.), 3 ml of scintillation cocktail (5 g PPO and 0.3 g POPOP/l toluene) were added to each vial, and then the radioactivity was measured with a Beckman LS-8000 liquid scintillation counter.

Protein was measured according to the method of Lowry *et al.* (9), as modified by Miller (13).

Lipid Identification. The phospholipids were identified by

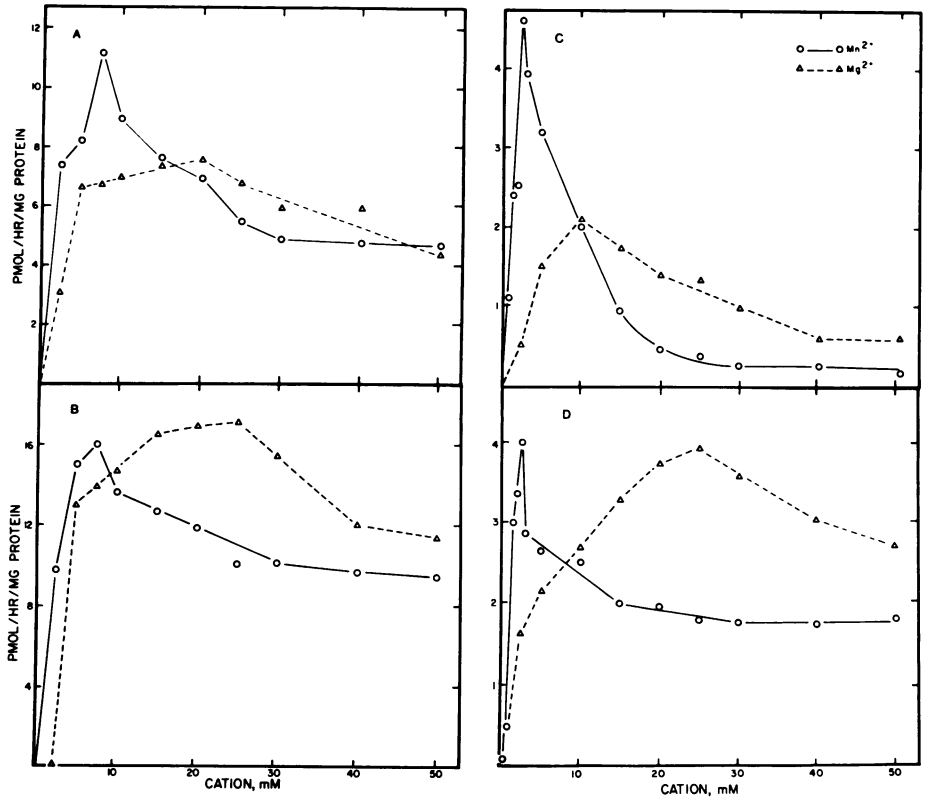

FIG. 1. Microsomal (A, B) and mitochondrial (C, D) cytidyltransferase activity in the absence (A, C) or presence (B, D) of phosphatidate (egg) and the presence of Mn^{2+} (O——O) or Mg^{2+} (Δ---Δ). All other factors were as described in "Materials and Methods."

cochromatography with standards on silica gel G plates developed in chloroform-methanol-acetic acid-water (50:25:7:3, v/v).

RESULTS

Protein and Time Effects. The reaction utilizing microsomal enzyme was linear for up to 80 min, the longest time period tested. Increases in activity were proportional to added protein between 30 and 140 μg, but below 30 μg a disproportionately lower activity was noted. Similar results were obtained with the mitochondrial enzyme, which also exhibited a lag followed by a linear increase in activity up to 100 μg of protein. The reason for this lag was not examined.

pH and Cation Responses. The optimal pH for the microsomal reaction was 6.5 and the mitochondrial was 6.0, similar results being obtained with both Mes and Hepes buffers.

There was an absolute requirement for a divalent cation with each organelle enzyme, with both Mg^{2+} and Mn^{2+} stimulating activity (Figs. 1, A–D). The exact response of the microsomal enzyme depended upon the presence of phosphatidate. Without exogenous phosphatidate, the maximum specific activity in the presence of Mn^{2+} was approximately 2-fold that obtained with Mg^{2+} (Fig. 1A). On the other hand, the maximum specific activities with the two cations were similar in the presence of phosphatidate (Fig. 1B). The optimal concentrations were about 7.5 mM $MnCl_2$ and 20 to 25 mM $MgCl_2$ in either the presence or absence of added phosphatidate.

The mitochondrial enzyme gave maximal activity at about 2.5 mM Mn^{2+}, and 25 mM Mg^{2+} in the presence of exogenous phosphatidate (Fig. 1C). The maximum activity obtained with each cation was about the same in the presence of the lipid substrate. In the absence of added phosphatidate, the optimal Mg^{2+} concentration was 10 mM, and the activity was only about one-half that in the presence of 2.5 mM Mn^{2+} (Fig. 1D). Mixtures of the two cations resulted in no visible additive effects (Kleppinger-Sparace and Moore, unpublished data).

Detergent Effects. Both Triton X-100 and deoxycholate strongly inhibited the microsomal cytidyltransferase activity (Fig. 2A) at concentrations as low as 0.01% (w/w). The results were the same in the presence or absence of added phosphatidate. The mitochondrial activity was inhibited at 0.01% (w/w) Triton X-100 but stimulated at higher concentrations (Fig. 2B). The maximum increase in activity in the presence of phosphatidate was found at 0.06% (w/w) Triton X-100, where activity was stimulated 2.5-fold over the controls. Stimulation also was found in the absence of phosphatidate. Sodium deoxycholate inhibited the enzyme at all concentrations tested.

Substrate Effects. The microsomal enzyme appears to have access to some endogenous phosphatidate, since activity is apparent without additions (Fig. 3A). On the other hand, exogenous phosphatidate obtained from egg lecithin stimulated the activity. The mixed phosphatidates from egg lecithin stimulated activity greater than either dioleoyl or didecanoyl phosphatidates, with both the latter forms reducing activity at most of the concentrations tested.

Increasing CTP concentrations (Fig. 4A) resulted in two distinct slopes of ER activity, and saturation was not apparent at concentrations up to 500 μM (Kleppinger-Sparace and Moore, unpublished data). A concentration of 120 μM was chosen for routine assays because of the plateau obtained at that concentration. If the initial curve is adjusted using a constant 12% slope for the second increase, a typical Michaelis-Menten substrate curve saturating at above 100 μM is obtained (data not given). Estimates of the apparent K_m with either adjusted or unadjusted data yield a value of 16.7 μM. Addition of NaF to the assay, to inhibit any CTPase activity (20), had no effect on the reaction.

Some mitochondrial activity occurred in the absence of phosphatidate, but up to a 6-fold increase resulted from its addition (Fig. 3B). The specific effect was related to the fatty acid composition of the substrate, with phosphatidate derived from egg phosphatidylcholine giving a maximum stimulation between 0.5 to 2 mM. The didecanoyl form did not reach that level of

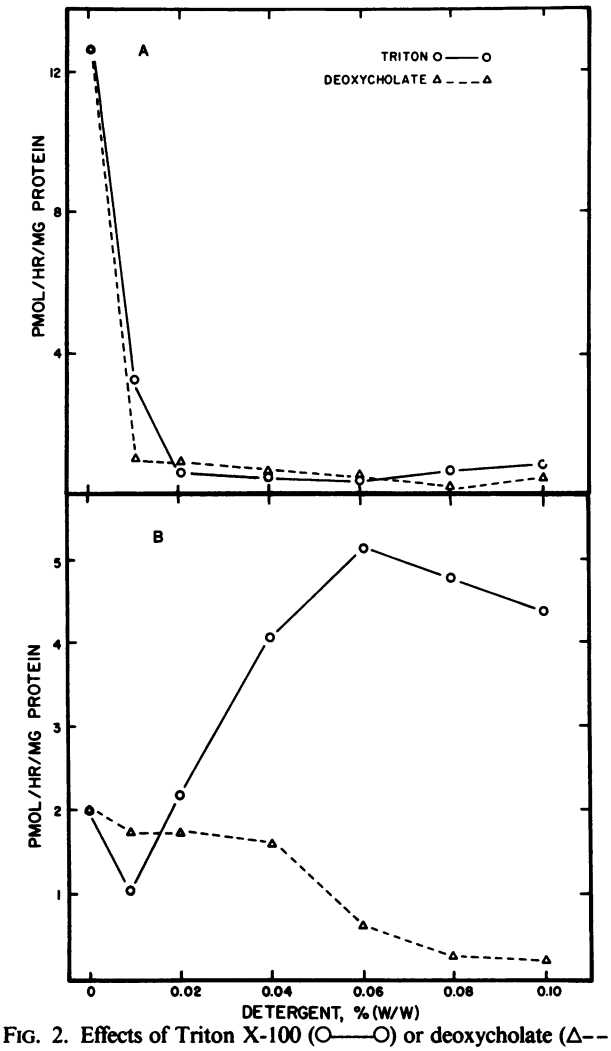

FIG. 2. Effects of Triton X-100 (O——O) or deoxycholate (Δ- - -Δ) on activity of the cytidyltransferase from ER (A) and mitochondria (B). All other factors were as described in "Materials and Methods."

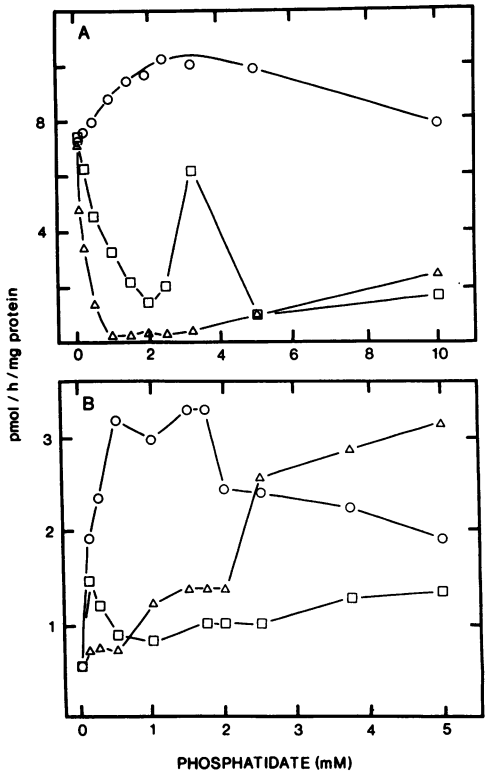

FIG. 3. Effects of various concentrations of the phosphatidate species didecanoyl (Δ——Δ), dioleoyl (□——□), or a mixture obtained from egg lecithin (O——O) on microsomal (A) and mitochondrial (B) cytidyl transferase activity. All other factors were as described in "Materials and Methods."

stimulation until about 4 to 5 mM and dioleoyl had little effect at concentrations as high as 5 mM.

Increasing CTP concentrations led to increased activity up to about 300 to 400 μM, in either the absence or the presence (Fig. 4B) of Triton X-100. The Michaelis constant doubled from 33.3 to 66.7 μM upon addition of the detergent, and possible substrate inhibition (or Mn^{2+} chelation) by CTP concentrations above 400 μM was observed.

DISCUSSION

This characterization of CTP:phosphatidate cytidyltransferase completes the general characterization of the synthetic pathways for phosphatidylglycerol and phosphatidylinositol in castor bean endosperm (15). The apparent K_m of the microsomal enzyme for CTP is lower than those reported for other plant microsomal enzymes (15), but the mitochondrial activity is about the same (1). Previous attempts to demonstrate stimulation of this enzyme activity by phosphatidate in plant tissues were negative (1, 20), but a 40% increase was found in this case for the microsomal activity and a 5-fold increase in the mitochondria. The differences might be related to differences in phospholipase D activity, which we have not detected in the castor bean endosperm (Kleppinger-Sparace and Moore, unpublished data), but which often is found in plant tissues.

The only previous report on the divalent cation requirement of plant microsomal cytidyltransferase was for one concentration and detected no differences in stimulation by Mg^{2+} or Mn^{2+}. Manganese was preferred by the castor bean enzyme. For the mitochondrial enzyme, previous workers found optimal Mn^{2+} concentrations ranging from 1 to 15 mM (15), which is similar to the 7.5 mM found in this investigation. For Mg^{2+}, the values range from 10 to 30 mM (15), as compared to the 25 mM in the absence of phosphatidate and 10 mM in its presence found here.

The mitochondrial enzyme was less susceptible than the microsomal enzyme to inhibition by either deoxycholate or Triton X-100, and actually was stimulated by the nonionic detergent. Whether this stimulation is due to a direct effect on the enzyme or results from an increased permeability of the mitochondria to the substrates is uncertain. A similar stimulation by Triton X-100 has been reported by Sumida and Mudd (20) for activity from cauliflower inflorescence mitochondria, as well as certain mammalian mitochondria (16) and bacteria (11).

The CTP:phosphatidate cytidyltransferase of the ER and mitochondria from castor bean endosperm may be isozymes. This possibility is supported by their different responses to divalent cations, detergents, and substrates. Differences in properties also have been used to argue in favor of different enzymes for phosphatidylcholine and phosphatidylethanolamine synthesis (15) and different CDP-choline:diacylglycerol cholinephosphotransferase enzymes for the ER and mitochondria of the castor bean endosperm (18). Other interpretations are possible, however, and a definitive statement on these possibilities awaits purification and characterization of the enzymes involved.

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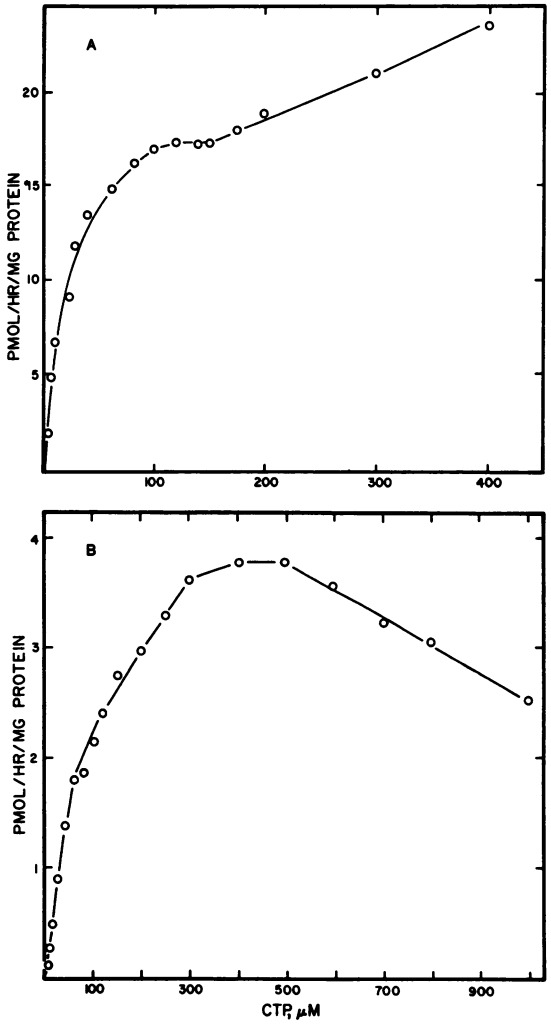

FIG. 4. Effect of increasing concentrations of CTP on the synthesis of CDP-diacylglycerol by the cytidyltransferase from the ER (A) and mitochondrial (B) fractions. The assay conditions were as described in "Materials and Methods."

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