Evidence that Phosphatidylcholine and Phosphatidylethanolamine are Synthesized by a Single Enzyme Present in the Endoplasmic Reticulum of Castor-Bean Endosperm

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Increasing concentrations of CDP-choline progressively inhibit the measured incorporation of CDP-[2-14C]ethanolamine into phosphatidylethanolamine catalysed by the ethanolaminephosphotransferase present in endoplasmic- reticulum membranes isolated from castor-bean endosperm cells. This inhibition parallels that observed during CDP-[Me-14C]choline incorporation and suggests that a single enzyme utilizes both these substrates.

The major structural phospholipids in the membrane fractions of castor-bean endosperm cells, in common with other eukaryotic cellular membranes, are phosphatidylcholine and phosphatidylethanolamine (Donaldson et al., 1972). The principal pathways for the incorporation of the bases choline and ethanolamine into phospholipids involve analogous reactions (Kennedy, 1961), with the ultimate step involving the reaction between CDP-choline or CDP-ethanolamine with a 1,2-diglyceride to form the respective phospholipid and CMP. These reactions occur in the microsomal fraction of animal and plant cells (Wilgram & Kennedy, 1963; Lord et al., 1973; Bowden & Lord, 1975). Several attempts have been made to distinguish whether separate enzymes are present in the microsomal fraction to synthesize these phospholipids or whether the cholinephosphotransferase and ethanolaminephosphotransferase activities reside in a single protein. Early work by Kennedy (1956) suggested that, in rat liver, the enzyme synthesizing phosphatidylcholine was more stable to freeze-drying than that synthesizing phosphatidylethanolamine, whereas other studies have indicated that a single enzyme may catalyse the synthesis of both phospholipids (Chojnacki, 1964; Macher & Mudd, 1974).

The present paper deals with a study of this question using endoplasmic-reticulum membranes isolated from castor-bean endosperm tissue, and provides further evidence that a single enzyme is responsible for phosphatidylcholine and phosphatidylethanolamine synthesis.

Experimental

Preparation of endoplasmic-reticulum membranes. Seeds of castor bean (Ricinus communis) were soaked overnight in running tap water and germinated at 32°C in moist vermiculite in darkness. Endosperm tissue, removed from 3-day-old seedlings, was homogenized by chopping with a single razor blade and cellular organelles were separated by sucrose-density-gradient centrifugation as previously described (Lord et al., 1972). After centrifugation, the endoplasmic-reticulum membranes were recovered as a discrete band in the sucrose gradient at a mean buoyant density of 1.12 g/cm3 (Lord et al., 1973). Gradient fractions (1.0ml) were collected by using a Beckman gradient fractionator, and those containing endoplasmic-reticulum membranes were pooled and used as the enzyme source (Bowden & Lord, 1975).

Enzyme assays. Cholinephosphotransferase (EC 2.7.8.2) activity was determined in a reaction mixture containing, in a final volume of 1.0ml, 15mM-Tricine, pH 7.5, 10mM-MgCl2, 0.33 μM-CDP-[Me-14C]choline (0.02 μCi) and enzyme. The mixture was incubated at 25°C and the reaction stopped by the addition of 2.0ml of ethanol. The precipitated protein was removed by centrifugation. The ethanol phase was mixed with 2.0ml of chloroform, and unchanged CDP-[14C]choline was removed by washing twice with 2M-KCl and twice with water. The organic phase was transferred to a scintillation vial and evaporated to dryness; 10ml of scintillator (Bray, 1960) was added and radioactivity was determined in a Packard Tri-Carb liquid-scintillation counter. Ethanolaminephosphotransferase (EC 2.7.8.1) was assayed exactly as described above but by replacing the CDP-[14C]choline with 0.7 μM-CDP-[2-14C]ethanolamine (containing 0.02 μCi). When appropriate, CDP-choline was added to reaction mixtures to give the final concentrations indicated in Figs. 1 and 2.

Reagents. CDP-[Me-14C]choline (28 Ci/mol) and CDP-[2-14C]ethanolamine (60 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and CDP-choline was from Serdary Research Laboratories, London, Ont., Canada.
Results and discussion

The radioactivity (c.p.m.) recovered in phosphatidylcholine during the standard cholinephosphotransferase assay over a 30 min reaction period was decreased by diluting the CDP-[14C]choline with increasing concentrations of unlabelled CDP-choline (Fig. 1a). Although calculations of the amount of phosphatidylcholine formed, based on the specific radioactivity of CDP-[14C]choline, established that increased amounts of phospholipid are formed with increasing CDP-choline concentration up to 100 μM, this decrease in measurable phospholipid radioactivity was expected. Virtually identical results were obtained when increasing concentrations of CDP-choline were added to ethanolaminephosphotransferase assays (Fig. 1b). This observation suggests that CDP-choline serves either as a second substrate for ethanolaminephosphotransferase or as an inhibitor of this enzyme. The similarity in the magnitude of this effect (Fig. 1b) to that of CDP-choline on the choline-phosphotransferase assays (Fig. 1a) favours the conclusion that a single enzyme is responsible for the synthesis of both phosphatidylcholine and phosphatidylethanolamine in the endoplasmic- reticulum fraction of castor-bean endosperm cells. Similar results and conclusions have been obtained from studies of these enzymic activities in spinach (Macher & Mudd, 1974).

The effect of increasing CDP-choline concentration on phosphatidylcholine synthesis is shown in Fig. 2.
Assuming that CDP-choline and CDP-[14C]ethanolamine formed a single CDP (base) pool available to the enzyme, an identical substrate concentration curve was obtained when increasing CDP-choline concentrations were added to the ethanolamine-phosphotransferase assays (Fig. 2). Lineweaver–Burk plots of these results (not shown) gave good agreement between the Michaelis constants for CDP-base for the cholinephosphotransferase and ethanolaminephosphotransferase activities of the microsomal enzyme, values of 7.3 and 7.8 μM respectively, with maximum velocities of 3.85 and 3.65 nmol/h.

If the rate with substrate 1 (S1) is v1 and the rate with substrate 2 (S2) is v2, then, assuming a single enzyme utilizes both substrates, the rates in the presence of the alternative substrates will be:

\[
v_1 = \frac{V_{\text{max},1}[S_1]}{[S_1] + K_{m,1} \left(1 + \frac{[S_2]}{K_{m,2}}\right)}
\]

\[
v_2 = \frac{V_{\text{max},2}[S_2]}{[S_2] + K_{m,2} \left(1 + \frac{[S_1]}{K_{m,1}}\right)}
\]

Rates were calculated from the above equations for a reaction mixture including 0.7 μM-CDP-[14C]ethanolamine (S1) and 1.25 μM-CDP-choline (S2). Calculated values were 0.27 nmol/h for S1 and 0.525 nmol/h for S2, i.e. a total rate for CDP-base (v1 + v2) of 0.795 nmol/h, which was in good agreement with the experimentally determined value of 0.80 nmol/h. This agreement between the calculated values and the experimental findings is consistent with a single enzyme using both substrates. The possibility that two enzymes are involved cannot be ruled out, but would require the somewhat unlikely situation that \( K_{m,1} \) for S1 acting as a substrate for enzyme 1 should equal the \( K_i \) for S1 acting as an inhibitor of enzyme 2, and a similar relationship must hold for \( K_{m,2} \).

The studies by Kennedy (1961) established that the major pathways for the incorporation of choline and ethanolamine into phospholipids involve initially the phosphorylation of the two bases, and a single cytoplasmic enzyme, choline kinase (EC 2.7.1.32), has been reported to catalyse the phosphorylation of both choline and ethanolamine (Wittenberg & Kornberg, 1953; Hanbrich, 1973). More recently, Broad & Dawson (1974) have provided different lines of evidence that in the rumen protozoan Entodinium caudatum the two bases are phosphorylated by different enzymes. Distinct kinase enzymes to initiate the CDP-base sequence may indicate distinct pathways, including the ultimate reactions in the endoplasmic reticulum, are involved in the synthesis of phosphatidylcholine and phosphatidylethanolamine. The present findings suggest that this is not the case in castor-bean endosperm, where a single enzyme apparently possesses cholinephosphotransferase and ethanolaminephosphotransferase activities. Both these phospholipids are exclusively synthesized by the endoplasmic-reticulum fraction from this tissue (Lord et al., 1973; Bowden & Lord, 1975), and developmental changes in the capacity of the tissue to synthesize phosphatidylcholine are paralleled exactly by its capacity to synthesize phosphatidylethanolamine (Bowden & Lord, 1975).

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