The intracellular distribution and catalytic properties of CTP:ethanolaminephosphate cytidylyltransferase from endosperm of castor bean (Ricinus communis L. var. Hale) have been studied. This enzyme was confined to membranes, with about 80% of the activity occurring in mitochondria and the rest in endoplasmic reticulum (ER) following sucrose density gradient centrifugation. The mitochondrial location of this enzyme was supported by further purifying mitochondria on Percoll density gradients. The mitochondrial cytidylyltransferase was detected largely in outer membrane fractions, and lost its activity after trypsin treatment, indicating that the active sites are exposed to the cytoplasm. Both mitochondrial and ER cytidylyltransferase required cations for activity; Mg²⁺ was preferred over Mn²⁺ and Ca²⁺. The pH optima both were 6.5. The apparent Kₐ values for ethanolamine phosphate were 143 and 83 μM and those for CTP were 125 and 1010 μM, respectively, for the mitochondrial and ER activities. The mitochondrial cytidylyltransferase reached a maximal velocity of 3.0 nmol/min/mg protein, whereas ER cytidylyltransferase was 0.424 nmol/min/mg protein. These findings reveal that the majority of the cytidylyltransferase activity in castor bean endosperm is not closely associated with ethanolaminephospho-transferase (predominantly in ER) which catalyzes the subsequent reaction in the synthesis of phosphatidylethanolamine by a nucleotide pathway. The possible roles of these enzymes in phosphatidylethanolamine synthesis in plants are discussed.

The synthesis of phosphatidylethanolamine (Ptd·Etn),¹ a major phospholipid of eucaryotic cells, can occur through the pathways of decarboxylation of Ptd-serine (1), exchange of ethanolamine with the head groups of other phospholipids (2), and transfer of Etn·P from CDP·Etn to DAG in a manner analogous to that of the nucleotide pathway of Ptd-choline synthesis (3). The Ptd-serine decarboxylase, base exchange enzyme, and Etn·P transferase have been well documented in several plant species (4, 5). The CDP·Etn pathway has been proposed to be dominant in the total Ptd·Etn biosynthesis of plants (4, 6) and such dominance also has been reported in some mammalian systems (7). The relative contribution may vary, however, with the CDP·Etn pathway contributing more to the total Ptd·Etn synthesis when exogenous ethanolamine is present (7-9).

CTP·Etn·P cytidylyltransferase (EC 2.7.7.14.) catalyzes a central reaction in the nucleotide pathway. This enzyme has received little attention in the literature, but is thought to play an important role in regulating Ptd·Etn synthesis by the nucleotide pathway in mammalian tissues (7), from which it has been reported to be totally soluble (10). The synthesis of CDP·Etn in plants has not been characterized. The occurrence of this activity in carrot was only briefly mentioned in a 1956 report (3), and no properties of this enzyme were described. Subsequent experiments with spinach leaves in other laboratories failed to detect the enzyme (11-13).

In our recent characterization of the substrate specificity of purified choline·P cytidylyltransferase (14), Etn·P cytidylyltransferase was found in cell-free extracts of castor bean endosperm. CDP·Etn has been detected in castor bean after incubation of endosperm with [³⁵S]serine (15). Serine has been suggested as the source for Etn·P synthesis in mammals (16) and so could serve to produce the substrate for the cytidylyltransferase. In order to gain a better understanding of this enzyme, and thereby Ptd·Etn synthesis by the nucleotide pathway in plants, we have further investigated the intracellular distribution and properties of the Etn·P cytidylyltransferase. The results reported here have shown that the majority of CTP·Etn·P cytidylyltransferase occurs in the mitochondria, associated largely with the outer membrane, and approximately 20% of the activity is in the ER. This intracellular distribution is in contrast with that of Etn·P transferase which is predominantly located in the ER (17).

EXPERIMENTAL PROCEDURES

Materials

Seed coats were removed from seeds of castor bean (Ricinus communis L. var. Hale) before inhibition. Endosperm halves were collected after germination for 2 or 3 days at 30 °C (18). Radioisotopes and other reagents were obtained from the sources reported (19). The [2-¹⁴C]Etn·P was either a product of Amersham (in 1982 and stored at −20 °C; no longer commercially available) or synthesized from [2-¹⁴C]Etn by Etn kinase of castor bean endosperm (see below). [2-¹⁴C]Etn·P was produced from [²⁻¹⁴C]Etn and ATP by an Etn kinase reaction. The procedure was modified from that used for soybean seeds (20). Endosperm halves, 2 days after inhibition, were frozen in liquid nitrogen, ground into fine powder, and suspended in 1.5 volume of a buffered solution containing 25 mM Tris·HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 5 mM DTT (buffer A). The slurry was centrifuged at 120,000 × g for 1 h, following which the supernatant was passed through a Sephadex G25 column and the protein eluate used as the source of Etn kinase.

The incubation mixture for Etn·P synthesis contained 50 mM Tris·HCl (pH 8.3), 3 mM ATP, 3 mM MgCl₂, 665 μM [²⁻¹⁴C]Etn (1.58 GBq/μmol), and 300 μg of protein in a final volume of 1 ml. The reaction was incubated at 30 °C for 30 min and terminated by adding

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¹ The abbreviations used are: Ptd, phosphatidyl; Etn, ethanolamine; DAG, diacylglycerol; DTT, dithiothreitol; Mes, 3-(N-morpholino)propanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; TLC, thin layer chromatography; ER, endoplasmic reticulum.
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80 μl of 60% trichloroacetic acid. The acid-precipitated proteins were removed by centrifugation. To assess the synthesis of Etn-P from Etn, an aliquot (2 μl) was spotted on a strip of Whatman 3 MM chromatography paper, and an ascending paper chromatogram was developed in ethanol:isopropanol:NH₄OH (6:5:2:0.3:3:5, v/v) (20). The Etn bands were detected by ninhydrin. Etn-P stayed at the origin and Etn migrated at an Rᵢ of 0.6. Approximately 75% of the Etn was converted to Etn-P in a typical reaction.

The reaction mixture was concentrated to about 150 μl with a Speed-Vac, and Etn-P was separated from Etn by the ascending paper chromatography method described above. Etn-P was eluted from the paper strips with water and lyophilized. The purity of Etn-P was verified by TLC based on the method of Sandler (10). An aliquot of dissolved Etn-P was applied to a TLC plate (Silica gel G), which was then developed with 96% ethanol-3% NH₄OH (1:2, v/v). This system gave an excellent separation of CDP-Etn, Etn-P, and free Etn with their Rᵢ values being 0.85, 0.57, and 0.35. Little free Etn or CDP-Etn was detected in the eluted Etn-P. In some experiments, Etn-P was further purified on a column of AG 1-X8 (formate form) according to the method of Sandler (9).

The original specific radioactivity of [2-¹⁴C]Etn was used to calculate the concentration of the synthesized Etn-P since the Etn and Etn-P endogenous to the castor bean extracts had been removed by the Sephadex G25 gel filtration.

Tissue Homogenization and Centrifugation—All the following procedures were carried out at 4 °C unless stated otherwise.

30 endosperm halves, 3 days after inhibition, were homogenized by chopping for 15 min with a razor blade in 10 ml of homogenization medium containing 0.5 mM sucrose, 0.15 M Tricine-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, and 10 mM DTT. The resultant homogenate was filtered through two layers of nylon cloth. For differential centrifugation studies the filtrate was successively centrifuged at 2700 g, 19,000 g, and 100,000 g., for 15 min. The homogenized mitochondria were diluted with the 10 ml of homogenization medium containing 0.5 mM sucrose, 0.15 M Tricine-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, and 10 mM DTT. The resultant homogenate was filtered through two layers of nylon cloth. For differential centrifugation studies the filtrate was successively centrifuged at 2700 g, 19,000 g, and 100,000 g., for 15 min. The homogenized mitochondria were diluted with the 10 ml of homogenization medium containing 0.5 mM sucrose, 0.15 M Tricine-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, and 10 mM DTT.

Submitochondrial fractions were prepared according to the procedure of Maisterrena (23) as modified by Sparsé and Moore (24). Intact mitochondria isolated from sucrose gradients were diluted and then suspended in an equal volume of ice chilled water and pelleted at 20,000 × gₑ₀ for 15 min. The pellet was resuspended in a phosphate buffer (10 mM pH 7.4) and homogenized with a Potter homogenizer for 10 min. The homogenized mitochondria were diluted with the 10 ml of phosphate buffer to a protein concentration of 0.1 mg/ml and left for 20 min. The homogenate was centrifuged at 100,000 × gₑ₀ for 1 h and the pellet was resuspended in 8.6% (w/v) sucrose. This resuspended material was layered onto a step sucrose gradient consisting of 61.5 (5 ml), 51.7 (5 ml), 37.7 (10 ml), and 25.2% (10 ml) sucrose in 10 mM Tris-HCl (pH 7.4). The gradients were centrifuged at 96,000 × gₑ₀ for 2 h in a Sorvall AH-629 rotor and then fractionated for assaying protein, marker enzymes, and Etn-P cytidyltransferase.

To further purify mitochondria on Percoll density gradients, mitochondria isolated from sucrose gradients were diluted with 5 volumes of washing medium containing 300 mM mannitol, 10 mM MOPS (pH 7.5), 1 mM EDTA, and 0.2% (w/v) bovine serum albumin and centrifuged at 15,000 × gₑ₀ for 15 min. The pellet mitochondria were resuspended with a Potter homogenizer (3 strokes) in 5 ml of the washing medium and the suspension layered on top of 30 ml of medium containing 300 mM mannitol, 10 mM MOPS (pH 7.5), 1 mM EDTA, bovine serum albumin and 30% Percoll (v/v). The tubes were centrifuged at 75,000 × gₑ₀ for 30 min, after which two bands were observed, one dense band nearly 2 mm from bottom of the tube and a lighter band at the interface between the sample suspension and Percoll gradient. The gradients were fractionated for assaying the Etn-P cytidyltransferase and various marker enzymes and the dense band was found to contain mitochondria essentially devoid of contamination by other cellular membranes. In later experiments, the mitochondria were removed from the Percoll gradients by using a pipette, diluted with 5 volumes of washing medium, and centrifuged at 15,000 × gₑ₀ for 15 min to remove Percoll. The purified mitochondria were suspended in washing medium.

Trypsin Treatment—The pelleted intact mitochondria collected from sucrose density gradients were suspended in buffer B to a final protein concentration of 1 mg/ml. Trypsin was added at 100 μg/mg mitochondrial protein, and the mixture was incubated at 30 °C for 20 min. The digestion was terminated by adding trypsin inhibitor to three times the amount (w/w) of trypsin present (25). The mixture was then incubated at 30 °C for another 15 min and centrifuged at 4 °C and 120,000 × gₑ₀ for 40 min. The mitochondrial pellet was resuspended in buffer B and assayed for various markers and Etn-synthesizing enzymes.

Enzyme Assays—For the Etn-P cytidyltransferase assay, the final reaction mixture (50 μl) routinely contained 1.8 mM CTP, 8 mM MgCl₂, 100 mM MES (pH 6.5), 3.12 μM [2-¹⁴C]Etn (1.58 GBq/ 

RESULTS

Validation of Enzyme Assay—The formation of CDP-Etn was assayed with a Norit charcoal method originally developed to measure CDP-choline in the choline-P cytidyltransferase assay (31). It is quicker and more convenient than chromatography methods. For the CDP-choline assay, binding of CDP-choline to charcoal is very specific and essentially no choline-P remains with the charcoal after washing. Unlike choline-P, about 2% of the total Etn-P remained bound to the charcoal even after extensive washing. This background occurred with both commercial and enzyme-synthesized Etn-P. This problem was overcome by using reactions without CTP as controls.

The synthesis of CDP-Etn as the product was confirmed by TLC and descending paper chromatography. Etn, Etn-P, and CDP-Etn were well separated by TLC (see “Experimental Procedures”) and no radioactivity was found in free Etn under normal assay conditions, indicating that hydrolysis of Etn-P by phosphatases in the assay was minimal. The use of paper chromatography, which was detailed in our previous report (14), also distinguished CDP-Etn from its methylated derivatives (i.e. CDP-methylEtn, CDP-dimethylEtn, and CDP-choline), and confirmed the absence of contamination by these analogues.

In many of the experiments, Etn-P cytidyltransferase was assayed in 156 pmol of Etn-P without adding nonradioactive carrier. To determine the validity of this condition, enzyme activity was examined as a function of protein concentrations and reaction time. A linear increase in activity occurred at protein concentrations tested up to 5 or 23 μg, respectively, for the mitochondrial and ER enzymes. Protein concentrations within the linear range were used for all subsequent enzyme characterizations. The enzyme activity also was linear
up to 90 min, the longest time tested.

Intracellular Distribution—Following differential centrifugation, 85% of the Etn-P cytidylyltransferase activity was associated with a 12,000 × g pellet and nearly all the activity sedimented by 100,000 × g (Table I).

In order to determine the specific subcellular location of this enzyme, organelles were first separated by sucrose density gradient centrifugation. Marker enzyme activities were used to determine the location of various cellular membranes (Fig. 1). The major peak of the Etn-P cytidylyltransferase activity (83% of total) across the gradients coincided with the peak of fumarase, a mitochondrial marker, and the rest of the cytidylyltransferase activity occurred at the peak of Etn-P transferase (Fig. 1) and choline-P transferase (data not shown), both of which have been reported to be associated with the ER in castor bean endosperm (4). The peaks of glucan synthase I and Triton-stimulated UDPase activities, which have been used as markers for Golgi membranes (30), overlapped extensively with the ER (Fig. 1). Little activity of either glucan synthase II or vanadate-sensitive ATPase (plasma membrane markers; 30) was detected in the major peak of the cytidylyltransferase (data not shown).

According to the distribution of the marker enzymes for ER (Etn-P transferase) and Golgi (glucan synthase I and Triton-stimulated UDPase), 18 and 20% of the ER and Golgi membranes, respectively, were present in the mitochondrial fractions from the sucrose gradients, as calculated by dividing the marker enzyme activities found to occur in the fumarase peak by the sum of their activities in all gradient fractions. Therefore, while some of the mitochondrial Etn-P cytidylyltransferase activity might be caused by ER contamination, this could only have accounted for about 3% of the total activity as calculated by multiplying the ER cytidylyltransferase activity (17%) and the portion of ER trapped in the mitochondrial fractions (18%). Thus, approximately 80% of the Etn-P cytidylyltransferase was estimated to be associated with mitochondria while 20% was with the ER.

It was recently reported that there exists in rat liver an ER subfraction which is closely associated with mitochondria and contains phospholipid-synthesizing enzyme activities (32). Therefore, it was deemed necessary to further purify the mitochondrial fraction in order to confirm that the Etn-P cytidylyltransferase was indeed inherent to mitochondria. Thus, the mitochondria isolated from the sucrose gradients were recentrifuged on Percoll gradients and fractions of the gradients were assayed for marker enzymes characteristic of several subcellular membranes (Fig. 2, Table II). At least 80% of the Etn-P cytidylyltransferase activity in the Percoll gradient was found in a dense band near the bottom of the gradient. The dense band was enriched with cytochrome c oxidase and fumarase activities (mitochondrial markers) and was almost completely devoid of Etn-P and choline-P transferases (ER markers), Triton-stimulated UDPase and glucan synthase I (Golgi markers), and vanadate-sensitive ATPase (plasma membrane marker). The enzyme activities typical of the ER and Golgi which were associated with the sucrose gradient-isolated mitochondria were recovered in a lighter band on the Percoll gradient (Fig. 2, Table II). These results indicate that highly purified mitochondria were obtained with the Percoll gradient centrifugation and confirm that the mitochondrial fractions contain the Etn-P cytidylyltransferase activity. The small amount of the cytidylyltransferase activity found in the upper band could be attributed partially to ER activity, but some was also due to residual mitochondria, as indicated by the presence of a small amount of fumarase and cytochrome c oxidase activities in this band (Fig. 2).

Submitochondrial Distribution—The submitochondrial location of Etn-P cytidylyltransferase was investigated by separation of the outer, ruptured inner, and intact inner mitochondrial membranes. The marker enzyme distributions (Fig. 3) were in agreement with those reported by Sparace and Moore (24). Two peaks of cytochrome c oxidase, an integral mitochondrial inner membrane enzyme, were found in dense membrane fractions 19 (1.14 g/cm²) and 29 (1.21 g/cm²), which were previously identified as being derived from the ruptured and intact inner membranes, respectively (24). This was supported by the observation that the peak of fumarase activity, a matrix enzyme, was found in the most dense region,

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**TABLE I**

**Subcellular distribution of ethanolamine-P cytidylyltransferase after differential centrifugation**

| Fraction | Specific activity (pmol/min/mg) | Total activity (pmol/min/ml) | Fraction of total activity (%) |
|----------|--------------------------------|----------------------------|-------------------------------|
| Homogenate | 4.1                            | 59.0                        | 100                           |
| 270 × g Sp | 6.4                            | 54.4                        | 92.2                          |
| 12,000 × g Sp | 1.5                        | 8.8                         | 14.8                          |
| 100,000 × g Sp | 0.6                        | 2.8                         | 4.8                           |

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**Fig. 1. Intracellular location of ethanolamine-P cytidylyltransferase and marker enzymes following linear sucrose gradient centrifugation.** Markers were used for the endoplasmic reticulum (ethanolamine-P transferase), Golgi (glucan synthase I and Triton-stimulated UDPase), mitochondria (fumarase), and glyoxysomes (catalase). The fraction numbers are from top to bottom of the gradient.
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The activities of NADH:cytochrome c reductase in both fractions was antimycin A-sensitive (Fig. 3). The outer mitochondrial membrane occurred in light density fractions (peaked at fraction 7, 1.10 g/cm³) where all the NADH:cytochrome c reductase activity was antimycin A-insensitive. The Etn-P cytidylyltransferase was found mainly in the outer and intact inner membrane fractions. The intact inner membrane fractions, however, appeared not to be completely free of contamination by outer membrane, as indicated by the presence of antimycin A-insensitive NADH:cytochrome c reductase in those fractions (Fig. 3). The activity ratios of the Etn-P cytidylyltransferase to antimycin-A insensitive NADH:cytochrome c reductase appeared to be the same (about 50) for both the outer and intact inner membrane fractions. Therefore, the cytidylyltransferase activity in the inner membrane fractions might be due to contamination by outer membranes.

The localization of the cytidylyltransferase in mitochondria was further examined by exposing intact mitochondria to trypsin (Table III). The outer mitochondrial membranes are generally permeable to molecules with molecular weights of several thousands, while the molecular weight of trypsin is about 26 kDa (25). Thus, trypsin is able to digest only the proteins exposed to the outer face of the outer mitochondrial membrane. Cytochrome c oxidase and fumarase were apparently protected from trypsin digestion due to the inability of the proteolytic enzyme to penetrate the mitochondrial membranes. On the other hand, NADH:cytochrome c reductase activity was partially lost, apparently because of exposure in the outer membrane. Etn-P cytidylyltransferase activity was almost completely eliminated by this treatment. These observations provide further evidence that the cytidylyltransferase is associated with the outer membrane, and indicate that its active sites are located on the cytoplasmic face.

**Catalytic Characteristics**—In order to obtain a first estimate of the similarities between the ER and mitochondrial activities, and the overall potential for activity within the cells, some general catalytic characteristics of the Etn-P cytidylyltransferases were measured. The enzyme from both the mitochondria and ER required cations for activity. Mg²⁺ was preferred to Mn²⁺ and Ca²⁺; Mn²⁺ and Ca²⁺ gave about 50% and 10%, respectively, of Mg²⁺ stimulated activity (Table IV), and the optimal Mg²⁺ concentration was above 2.0 mM (Fig. 4A). Some residual activity was observed in the 270 × g₀ supernatant even though Mg²⁺ was not included. This residual activity was most likely the result of a carryover of Mg²⁺ from the tissue, since it was abolished by including EDTA in the assay solution. The optimal pH for CDP-Etn synthesis by both the mitochondrial and ER membranes determined in the presence of Mg²⁺ was 6.5 (Fig. 4B).

The Michaelis-Menten constants for the enzyme from the mitochondria were slightly higher than those of the ER, with apparent Kₘ values toward Etn-P being 143 and 83 μM (Fig. 4C), and toward CTP being 125 and 101 μM, respectively, for

**Table II**

Activities of ethanolamine-P cytidylyltransferase (Etn-P CT) and marker enzymes in mitochondrial fractions isolated from sucrose and Percoll gradients

| Subcellular fraction | Marker enzyme | Sucrose gradient | Percoll gradient |
|----------------------|---------------|-----------------|-----------------|
|                      |               | Lower           | Upper           |
| Mitochondria         | Etn-P cytidylyltransferase | 82.3            | 91.3            | 26.5 |
| Mitochondria         | Cytochrome c oxidase     | 8.9             | 10.1            | 2.1  |
| Mitochondria         | Fumarase              | 3.6             | 4.9             | 0.88 |
| ER                   | Etn-P transferase       | 105.3           | 1.7             | 171.4|
| ER                   | Choline-P transferase   | 132.3           | 2.2             | 166.5|
| Golgi                | Glucan synthase I       | 197.8           | 2.8             | 325.0|
| Golgi                | Triton-stimulated UDPase| 12.6            | 0.57            | 17.5 |
| Plasma membrane      | Vanadate-sensitive ATPase| 1.4             | 0               | 2.5  |
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**FIG. 3.** Submitochondrial distribution of ethanolamine-P cytidyltransferase and marker enzymes following step sucrose density gradient centrifugation. Markers were included for the soluble matrix protein (fumarase), inner membrane (cytochrome c oxidase), and outer membrane (antimycin A-insensitive NADH: cytochrome c reductase). Cyt c, cytochrome c.

**TABLE III**

Effects of trypsin treatment on mitochondrial ethanolamine-P cytidyltransferase (Etn-P CT)

Intact mitochondria were preincubated in the presence and absence of trypsin (100 μg/mg protein) at 30 °C for 20 min; see "Experimental Procedures." The activities of phosphatidylethanolamine synthesizing and marker enzymes were measured with aliquots of the resuspended mitochondrial pellet. Cyt c, cytochrome c.

| Treatment, Trypsin | Etn-P CT | Fumarase | Cyt c oxidase | NADH:Cyt c reductase |
|--------------------|----------|-----------|---------------|----------------------|
|                    | pmol/min/mg | μmol/min/mg | μmol/min/mg | NADH/Cyt c reductase |
| -                   | -99.5     | 8.7       | 1.6          | 1.4                  |
| +                   | 4.5       | 10.5      | 2.0          | 1.4                  |

**TABLE IV**

Effects of cations on ethanolamine-P cytidyltransferase

Reaction mixtures contained 5 mM cations, EDTA, or both as indicated. Values of activity were averages of two experiments, within each of which duplicate assays were used.

| Treatment | Mitochondria | ER 270-g supernatant |
|-----------|--------------|-----------------------|
|           | pmol/min/mg  | -EDTA +EDTA           |
| No cation | 0.85         | 0.43                  |
| Mg²⁺      | 125.10       | 13.26                 |
| Mn²⁺      | 65.68        | 7.66                  |
| Ca²⁺      | 10.28        | 1.94                  |

The inhibition of Etn-P cytidyltransferase by choline-P was also tested. Nonradioactive choline-P added to 50 times the concentration of Etn-P in the assay mixture did not inhibit the reaction. Similarly, nonradioactive Etn-P had no effect on purified choline-P cytidyltransferase (data not shown).

**DISCUSSION**

Occurrence of Etn-P Cytidylyltransferase in Plants—A major uncertainty concerning operation of the CDP-Etn pathway for Ptd-Etn synthesis in plants has resulted from a paucity of data supporting the existence of Etn-P cytidyltransferase. This investigation provides direct support for the occurrence of this enzyme in castor bean endosperm. Thus, strong evidence now exists for the nucleotide pathway for Ptd-Etn synthesis in plants (Fig. 1). Etn kinase has been purified from spinach and soybean (4) and the final transferase has been demonstrated and characterized in spinach, castor bean, and other plant species (4). Previous attempts to measure Etn-P cytidyltransferase have not, however, been successful in spinach (11–13). The discrepancy between our results and the experiments with spinach may arise from the fact that the spinach assays used a 15,000 g supernatant as the enzyme source (11–13), and so the membranes containing the activity may have been discarded.

The castor bean Etn-P cytidyltransferases compare variously with the enzyme from rat liver (10). They showed the
same cation preference (Mg$^{2+} > $Mn$^{2+} > Ca$^{2+}$) and substrate specificity (i.e. do not use choline-P). The substrate affinity of the Etn-P cytidylyltransferase toward Etn-P and CTP was also similar to that of rat liver (the K_m values of liver enzyme for Etn-P and CTP were 65 and 55 $\mu$M, respectively). The pH optimum of rat liver cytidylyltransferase (7.5–9) is higher than that from castor bean endosperm. The marked difference between the rat liver and castor bean cytidylyltransferase is that the former is cytosolic while the latter is almost completely membrane bound.

**Transfers of Cytidyl Moieties to Etn-P and Choline-P Are Catalyzed by Different Enzymes**—In a previous study we showed that purified choline-P cytidylyltransferase has no activity with Etn-P (14). Likewise, Etn-P cytidylyltransferase is not inhibited by choline-P. Further evidence for two completely separate enzymes being involved in these two reactions lies in the distinctly different subcellular locations of the two enzymes. The majority of choline-P cytidylyltransferase activity was recovered in the ER (17), whereas major portions of Etn-P cytidylyltransferase were in the mitochondria.

**Implications of the Compartmentalization**—The ER has been proposed as the major, albeit not exclusive, site for Ptd-Etn synthesis in plants through the nucleotide pathway, since the majority of the Etn-P transferase is in this organelle (4). The finding reported here that the major portion of Etn-P cytidylyltransferase is associated with the mitochondrial fraction was unexpected in the light of the fact that 2% of the Etn-P transferase, at most, has been found in this organelle (4, 17, 33). It might be expected that if the Etn-P cytidylyltransferase were membrane bound, it would be associated with the membrane containing the Etn-P transferase in order to provide CDP-Etn as a substrate for PtdEtn synthesis. For the ER, under optimal in vitro assay conditions, the cytidylyltransferase exhibited a V_max of 424 pmol/min/mg protein, or about 60% of the V_max for the final transferase (33). The apparent K_m of the final transferase for CDP-Etn (8 $\mu$M) (33) was 10 times lower than that of the cytidylyltransferase. Although the pool sizes of Etn-P and CDP-Etn in castor bean endosperm are not known, pulse-chase experiments suggest that Etn-P should be more concentrated than CDP-Etn in this tissue (34). In rat liver, Etn-P concentrations were more than 10-fold higher than those of CDP-Etn (9). Thus, a simple comparison of the two enzymes of the ER suggests that the ER Etn-P cytidylyltransferase would be the rate limiting enzyme for the pathway. Thus, the possibility exists that Etn-P cytidylyltransferase is responsible for regulating the overall pathway in the ER, similar to the situation for choline-P cytidylyltransferase for Ptd-choline synthesis (35).

A similar comparison between the two enzymes in mitochondria results in a more perplexing situation since the Etn-P cytidylyltransferase demonstrated a V_max of 3.0 nmol/min/mg protein, whereas the Etn-P transferase was only 0.217 nmol/min/mg protein. This indicates that CDP-Etn in this organelle has the potential of being synthesized at a rate far exceeding the capacity of the Etn-P transferase. Therefore, at least a portion of the CDP-Etn from Ptd-Etn, synthesis in the ER might be derived from the mitochondria since CDP-Etn is soluble and would be rapidly available to the Etn-P transferase by diffusion. The reason for this compartmentalization remains elusive. One possibility is that there is an energy requirement for CDP-Etn synthesis, but this seems unlikely since an analogous reaction, the synthesis of CDP-choline, occurs predominately in the ER and has a similar V_max (17), and Ptd-choline is synthesized at a rapid rate in this tissue (4). It is conceivable that one of the enzymes is subject to an independent regulatory mechanism in order to allow for excess production of CDP-Etn under specific conditions, but such conditions have yet to be defined. Another possibility is that the mitochondrial reaction contributes to a separate intracellular pool of CDP-Etn with a separate role. Pulse-chase experiments with castor bean hypocotyls suggest that choline derived from different precursors might be compartmentalized and used for different sets of metabolic reactions (34). Likewise, it has been reported in yeast and mammalian tissues that separate pools of water-soluble phospholipid precursors exist (36–38). Such pools could serve to feed unrelated pathways or as reserve supplies for regulating normal phospholipid synthesis in single or separate compartments. One intriguing alternative possibility for the mitochondrial cytidylyltransferase could include a mitochondrial role in production of N-acyl derivatives of Ptd-Etn, a form which makes up as much as 13% of the lipid phosphorous of some plant tissues (39).

Finally, it could be proposed that the reverse reaction of the cytidylyltransferases, leading to the formation of Etn-P and CTP from CDP-Etn and pyrophosphate, could be the significant activity of one or the other of these enzymes, perhaps playing a role in Ptd-Etn degradation. In our preliminary studies, the reactions catalyzed by both the mitochondrial and ER cytidylyltransferases appeared to be highly reversible (data not shown). Mitochondria are known to be the site of synthesis of Ptd-Etn by decarboxylation of Ptdserine in mammalian tissues (1). It might be speculated that excess Ptd-Etn synthesized in mitochondria could be metabolized by the reverse reactions of the nucleotide pathway enzymes, thereby generating Etn-P. This intermediate is potentially important in plants as a precursor of phosphocholine and so also Ptd-choline (40). However, it should be noted that although the synthesis of Ptd-Etn by decarboxylation of Ptdserine in plants is known to occur (4), the reaction has not been characterized and it is not known if the subcellular compartmentalization of this reaction in castor bean is analogous to that of the mammalian system. The requisite reversibility of the Etn-P transferase also remains to be confirmed. Whether the reversal of the reactions mentioned above is truly a result of cytidylyltransferase reversal or a completely separate enzyme must await purification of the enzyme(s) involved.

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