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Phosphatidylcholine Synthesis in Castor Bean Endosperm[1]

Free Bases as Intermediates

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ABSTRACT

The methylation steps in the biosynthesis of phosphatidylcholine by castor bean (*Ricinus communis* L.) endosperm have been studied by pulse-chase labeling. Endosperm halves were incubated with [*methyl*-^{14}C]*S*-adenosyl-L-methionine, [2-^{14}C]ethanolamine, [^{14}C]-ethanolamine phosphate, or [^{14}C]serine phosphate. The kinetics of appearance were followed in the free, phospho-, and phosphatidyl-bases. The initial methylation utilized ethanolamine as a substrate to form methylethanolamine, which was then converted to dimethylethanolamine, choline, and phosphomethylethanolamine. Subsequent methylations occurred at the phospho-base and, to a lesser extent, the phosphatidyl-base levels, after which the radioactivity either remained constant or decreased in these compounds and accumulated in phosphatidylcholine. Although the precursors tested did support the synthesis of choline, the kinetics of the labeling make them unlikely to be the major sources of free choline to be utilized for the nucleotide pathway. A model with two pools of choline is proposed, and the implications of these results for the pathways leading to phosphatidylcholine biosynthesis are discussed.

PtdCho[3] is the major membrane phospholipid in castor bean (*Ricinus communis* L.) endosperm (4, 9). During the postgermination period, levels of PtdCho vary independently of the other phospholipids (15, 21), which suggests that regulation of its synthesis may be complex. Two pathways have been considered as sources for PtdCho in this tissue: the nucleotide pathway in which PtdCho is synthesized from free choline and the methylation pathway in which PtdEtn is methylated to PtdCho (19). However, recent studies of the kinetics of methyl group incorporation into other plant tissues produced evidence that the synthesis of PtdCho involves a complex interrelationship between cytosolic and membrane-bound intermediates and that the methylation pattern differs from plant to plant. For spinach leaves, Coughlan and Wyn-Jones (5) suggested that the successive methylation of free Etn, MEtn, and DMEtn to form choline is the most important pathway. In *Lemna* (23) and salinized sugarbeet (10), all three methylations occurred at the phospho-base level, whereas in soybean, P-Etn was the substrate of the initial methylation, and the P-MEtn formed was used to form PtdEtn, which subsequently was methylated to form PtdCho (6). In water-stressed barley leaves (11) and in carrot (6), the methylation pathway for the biosynthesis of PtdCho was found to be more complex, because methylations took place at both the phospho-base and the phosphatidyl-base levels.

In castor bean endosperm, the nucleotide pathway of PtdCho synthesis is operative at a high level, as evidenced by the relatively high in vitro enzyme activities in comparison to the alternative phospholipid methylation pathway and the fact that exogenously administered Cho is immediately and readily available for PtdCho synthesis (13). However, the endogenous source of Cho is unknown. Ser has long been thought to be the precursor of Cho (8), but free Cho produced from exogenously added L-Ser appears unavailable for incorporation into PtdCho via the nucleotide pathway (13).

To understand better how PtdCho is synthesized and which compound is the source of Cho for the CDP-Cho pathway in castor bean endosperm, we followed the metabolic fates of ^{14}C-radiolabeled AdoMet, Etn, P-Etn, and P-Ser at the free and phospho-, as well as phosphatidyl, base levels. Some of the experiments have been reported in preliminary form (26).

MATERIALS AND METHODS

Plant Growth

Three-day postgermination endosperms of castor bean (*Ricinus communis* L. var Hale) were prepared as described previously (13).

Paper Chromatography

Except as otherwise noted, descending paper chromatography was carried out on Whatman No. 3 paper at room temperature. Solvents used were: solvent A, phenol:1-butanol:88% HCOOH:H_2O (125:125:6.8:25.7, w/v/v/v; to avoid tailing, the chromatography paper was predipped in 1 N KCl and dried, ref. 2); solvent B, 2-propanol:88% HCOOH:H_2O (7:1:2, v/v); solvent C, 2-propanol:29%

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[3] Abbreviations: Cho, choline; Etn, ethanolamine; MEtn, *N*-methylethanolamine; DMEtn, *N,N*-dimethylethanolamine. The phosphate esters of these compounds are designated by the prefix P (e.g. P-Etn, ethanolamine phosphate) and the corresponding phosphatidyl-base or CDP derivatives by the prefix Ptd or CDP, respectively. AdoMet, *S*-adenosyl-L-methionine.

$NH_4OH:H_2O$ (7:1:2, v/v) (19); solvent D, methanol:concentrated HCl:H_2O (9:1:1, v/v).

Radioactive Compounds

[*methyl*-^{14}C]Cho (50 mCi/mmol) was obtained from Amersham. [*methyl*-^{14}C]AdoMet (52 mCi/mmol) was purchased from ICN; before each experiment, its purity was tested by ascending paper chromatography with n-butanol:acetic acid:H_2O (60:15:25, v/v) as the developing solvent and was found never to be less than 93.5%. [2-^{14}C]Etn (57.3 mCi/mmol) and [^{14}C]P-Etn phosphate (49 mCi/mmol) were obtained from Amersham and purified by paper chromatography with solvent B. Eluted products were 97 to 98% and 92 to 94% pure, respectively.

[^{14}C]P-Ser was prepared (yield: 38 to 43%) from L-[3-^{14}C]-Ser (57 mCi/mmol, ICN) by a small-scale modification of the procedure of Neuhaus and Korbes (25). $POCl_3$ (111 μmol) and water (222 μmol) were incubated in a final volume of 14 μL for 2 h at 30°C with swirling every 10 min to facilitate removal of HCl. The monochlorophosphoric acid produced was added to L-[3-^{14}C]Ser (0.44 μmol) and dried under an N_2 stream. The reaction mixture was placed in a bath at 60°C for 4 h and hand-shaken periodically, following which 1 μL of water was cautiously added. The polyphosphates were hydrolyzed by addition of 4 μL of 1 N HCl, followed by heating in a boiling H_2O bath for 20 min. The solution was cooled to room temperature, and 11 μL of 95% ethanol were added, followed by 6 μL of ether. The [^{14}C]P-Ser synthesized was isolated from [^{14}C]Ser by chromatography in solvent B, which provided an excellent separation of these two compounds. Peaks of [^{14}C]P-Ser, located by counting an aliquot and visualized with ninhydrin reagent, were eluted twice with a mixture of methanol:H_2O (1:1, v/v). Paper chromatography with solvent B indicated that the P-Ser was 88% radiopure.

Labeling

Pulse-chase experiments were performed by treating endosperm halves (6) on the abaxial surface with 30 μL of [^{14}C]AdoMet (4 nmol/endosperm half) for 30 min or with [^{14}C]Etn (10 μL; 4 nmol/endosperm half) or [^{14}C]P-Ser (10 μL; 2 nmol/endosperm half) for 10 min. The specific compounds were diluted in 20 mM Hepes buffer at pH 7.0. From the total radioactivity incorporated into endosperm halves, it was calculated that [^{14}C]AdoMet, [^{14}C]Etn, [^{14}C]P-Etn, and [^{14}C]P-Ser had been taken up at 0.08, 0.32, 0.17, and 0.12 nmol·g^{-1} fresh weight min^{-1}, respectively. After the pulse, endosperm halves were washed with distilled H_2O for 2 min to remove external radioactive material. Incubation was then continued in a Petri dish with moistened paper for various additional times up to 8 h. Radiolabeled Etn was chased with cold Etn applied on the abaxial surface (80 nmol/endosperm half; 20 μL).

Extraction and Chromatography

Incubations were stopped by freezing the endosperm halves in liquid N_2. The frozen endosperms were ground to a fine powder with a mortar and pestle. This powder was extracted by a procedure similar to that described by Bieleski and Young (1) to minimize phosphatase activities. The powder was suspended with 20 mL (10 mL/g) of methanol:chloroform:0.2 M formic acid (12:5:3, v/v) previously cooled to dry-ice temperature for 2 h at −20°C. The homogenate was filtered under vacuum through a fiberglass filter disc. The residue on the filter was washed with 2 mL of methanol:chloroform:H_2O (12:5:3, v/v) and then with 1 mL of chloroform. The filtrate was adjusted to 20 mL with methanol:chloroform:H_2O (12:5:3) and 5 mL of chloroform were added, followed by 7 mL of H_2O. The resulting biphasic mixture was centrifuged 10 min at 5000 rpm in a benchtop centrifuge to separate the phases. The residue was homogenized twice for 30 s with 20 mL of ice-cold 0.2 M formic acid in 20% ethanol and then filtered. The filtrate and the methanol-H_2O phase were combined and evaporated to dryness in a rotary evaporator. This residue was taken up in a small volume of H_2O and initially chromatographed with solvent B (23). After elution twice in methanol:H_2O (1:1, v/v), the free bases, phosphorylated derivatives, and CDP-bases separated by the initial chromatography were chromatographed with solvent A (16 h), solvent C (64–112 h), and solvent D (16 h), respectively. Because castor bean endosperm contains high concentrations of triacylglycerols (9), lipids in the chloroform phase were first fractionated on a silicic acid column (60–200 mesh, Sigma) and eluted successively with chloroform, acetone, and methanol (28). Acetone eluates were separated on 250-μm silica gel G thin-layer plates in a two-dimensional solvent system consisting of chloroform:methanol:H_2O (65:15:2, v/v) and chloroform:acetone:methanol/acetic acid:H_2O (65:20:10:10:2, v/v) (17).

Phospholipids in the methanol fraction were chromatographed on silica gel TLC plates with chloroform:propionic acid:1-propanol:H_2O (3:2:6:1, v/v) in the first dimension and chloroform:methanol:acetic acid:H_2O (85:15:10:3.5, v/v) in the second. This latter chromatographic method provided excellent separations of PtdSer, PtdCho, PtdEtn, and the mono- and dimethyl derivatives of PtdEtn.

Under these conditions, the respective recoveries of authentic [^{14}C]PtdCho and [*methyl*-^{14}C]P-Cho (Amersham) added to unlabeled endosperm powder were 73 and 64%. Recoveries calculated for radiolabeled MEtn, 67%, and DMEtn, 64%, were similar to those for [^{14}C]P-Cho, indicating that these two free bases were not lost by volatilization during development, evaporation, or spotting.

Detection and Counting

In paper chromatography, authentic external standards were used to locate peaks of interest. [^{14}C]MEtn, [^{14}C]P-MEtn, and [^{14}C]P-DMEtn were prepared by methylating [^{14}C]Etn or [^{14}C]P-Etn with iodomethane (23). The identities of phosphorylated derivatives were confirmed by treatment with phosphatase followed by migration with solvent A chromatography of the free base released (23). The chromatograms were cut into 1-cm (solvents A, B, and D) or 0.5-cm (solvent C) segments, the radioactivity of which was measured directly in CytoScint (ICN) using a Beckman LS-8000 scintillation counter.

TLC plates were sprayed, after development, with primuline (34) and the polar lipids visualized under UV light. Spots were scraped from the plates for measuring radioactivity in ReadySolv (Beckman).

Total precursor uptake was estimated as the total amount of radiolabel incorporated into the combined residue, chloroform, and methanol-H_2O fractions. Incorporation of label into lipids and aqueous intermediates was expressed as a percentage of the total label incorporated into the tissue.

RESULTS

Metabolism of [^{14}C]AdoMet

The metabolic fate of the methyl group of AdoMet is shown in Figure 1. In the experiments of Datko and Mudd (6), the dominant utilization of methionine methyl groups was for synthesis of PtdCho. However, in castor bean endosperm, PtdCho was a minor product when compared with a compound that appeared in the acetone eluate (Fig. 1A). Similar results have been reported several times in the literature. Marshall and Kates (18) observed that in whole leaves and leaf slices of spinach the neutral lipid fraction was labeled from [^{14}C]AdoMet or [^{14}C]Ser but not from [^{14}C]Etn. In *Lemna*, Mudd and Datko (24) also reported a labeling in the neutral lipids when [^{14}C]Ser was the precursor. In castor bean endosperm, Kinney and Moore (13) reported that, when L-[^{14}C]-Ser was supplied, some labeled compound cochromatographed with the neutral lipids on TLC plates, and [^{14}C]-AdoMet was incorporated into unidentified lipids (15). The identities of these products remain unresolved, however. According to Kates (12), such acetone eluates may contain galactosyl diacylglycerols, cerebrosides, steryl glycosides, and sulfolipid plus small amounts of cardiolipin and phosphatidic acid. The compound obtained in the present study did not fluoresce with primuline following two-dimensional chromatography (17). It appeared not to be a steryl glycoside or phospholipid, because it did not react with perchloric acid or zindzadze reagent, respectively. Finally, with a HPLC method for separating all lipid classes, recently described by Moreau et al. (22), the compound eluted as a single peak but did not correlate with any of the standards used.

After the 30-min pulse, the major labeled product was mono-MEtn (Fig. 1B). By this time, radioactivity also had entered the two other Etn-containing free bases, DMEtn and Cho. Lesser amounts of radioactivity were found in the three methylated P-Etn derivatives, P-MEtn, P-DMEtn, and P-Cho (Fig. 1C). PtdCho (Fig. 1E) was already radiolabeled, whereas radioactivity in the methylated phosphatidyl intermediates, PtdMEtn and PtdDMEtn (Fig. 1D), was minimal. These data suggest that the initial methylation is of free Etn to form MEtn.

During the 1st h following the labeling period, the amount of radioactivity in the free bases increased at the same rate (Fig. 1B). This suggests that Etn was successively and rapidly methylated to MEtn, DMEtn, and then Cho. Radioactivity also increased in the phosphoryl-base, P-MEtn and P-Cho being radiolabeled more than P-DMEtn (Fig. 1C). Thereafter, radioactivity decreased in all of these compounds, whereas it accumulated in PtdCho. In PtdMEtn and PtdDMEtn, the label increased at 1 h but remained at a low level (Fig. 1D).

Four hours after the end of the pulse, there was no longer radioactivity in DMEtn, although some remained in MEtn, which continued to decrease (Fig. 1B). This suggests the

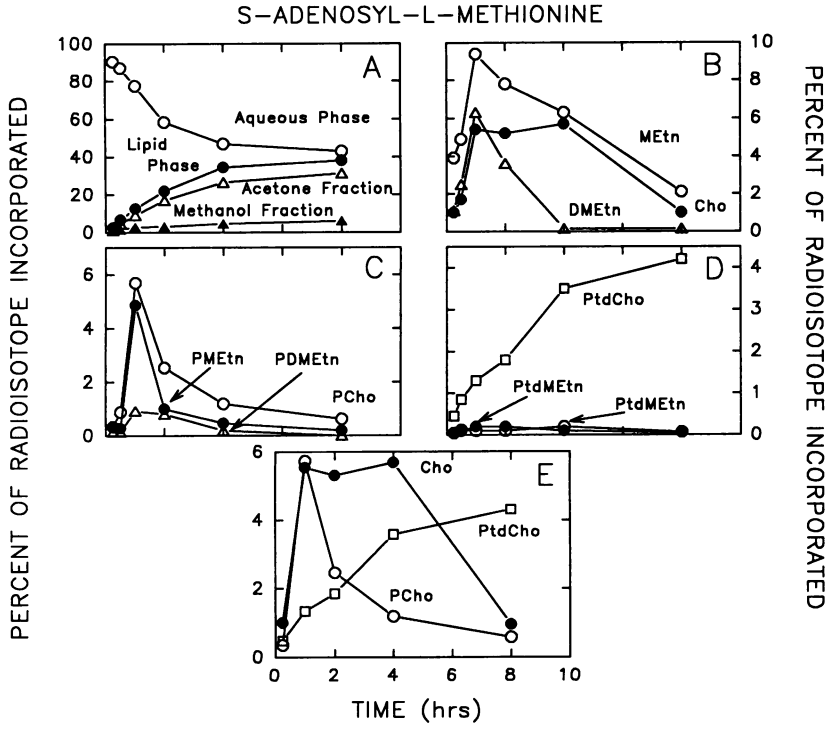

Figure 1. Radioactive products resulting from feeding castor bean endosperm a 30-min pulse with [*methyl*-^{14}C]AdoMet.

possibility that MEtn was partially methylated to DMEtn and partially converted to P-MEtn by the appropriate kinase.

The increased label in PtdCho appeared to coincide with the loss of label from P-Cho (Fig. 1E). No data were available for CDP-Cho, because this nucleotide was not separated from the precursor, AdoMet, by our paper chromatography methods. When, after 4 h, the radioactivity decreased in Cho, there was no further change in the labeling pattern of PtdCho. Although exogenously added AdoMet supported the synthesis of Cho, this Cho does not seem to be available for PtdCho synthesis.

Metabolism of [2-^{14}C]Etn

The results of the pulse-chase experiments with radiolabeled Etn are reported in Figure 2. When 4 nmol of [2-^{14}C]-Etn were supplied to castor bean endosperm halves, only P-Etn, CDP-Etn, and PtdEtn were labeled at the end of a 10-min pulse (Fig. 2F). Etn was then incorporated rapidly and in large quantities into PtdEtn, with this phospholipid accounting for more than 40% of the original label after 4 h. The amount of radioactivity in P-Etn also increased, but the P-Etn pool was saturated by 30 min. The CDP-Etn pool was saturated within 15 min after the pulse, and its size was very small, only traces of radioactivity being detected in this nucleotide.

Although PtdEtn was the major labeled product, Etn also entered PtdCho but only after a 15 min lag and in small quantities during the course of the experiment (Fig. 2E). Fifteen minutes after the end of the pulse, radiolabel was detected in the free bases MEtn, DMEtn, and Cho (Fig. 2B) and the phosphoryl-bases P-MEtn, P-DMEtn, and P-Cho (Fig. 2C), in greater quantities than in PtdCho (which was 0.10% at that time). The intermediate phosphatidyl-bases, PtdMEtn and PtdDMEtn, were also radiolabeled by 15 min after the pulse, but only a very small amount (Fig. 2D). Their labeling then increased in a parallel fashion, suggesting that some PtdDMEtn was synthesized by methylation of PtdMEtn. Methylations also seemed to occur at both the free base and phospho-base levels because the kinetics of labeling of MEtn, DMEtn, and Cho, on one hand, and of P-MEtn and P-DMEtn, on the other hand, were similar during the time of the experiment (Fig. 2, B and C).

Between 15 min and 2 h after the incubation period, the amount of radioactivity in P-MEtn and P-DMEtn decreased as label accumulated into P-Cho (Fig. 2C), PtdMEtn, and PtdDMEtn (Fig. 2D). This indicates the possibility of a source of label for the phosphatidyl-bases in addition to the successive methylations of PtdEtn, such as through the respective phosphoryl-bases via the nucleotide pathway.

The results confirmed the previous finding that the Etn that did not enter the base exchange and nucleotide pathways to form PtdEtn was rapidly methylated to form MEtn, DMEtn, and Cho. Whether the Cho participated in PtdCho synthesis via the nucleotide pathway is unclear because 4 h after the labeling period radioactivity accumulated again in free Cho as it decreased in P-Cho and increased in PtdCho.

Metabolism of [^{14}C]P-Etn

Because free Ser may not be available for PtdCho synthesis through the nucleotide pathway (13), the possibility that P-Ser could serve as the precursor of P-Cho was examined. If this pathway were operating, the first expected intermediate would be formed by decarboxylation of P-Ser to form P-Etn. For this reason, P-Etn was tested first.

Immediately after a 10-min pulse with [^{14}C]P-Etn, radioactivity appeared in Etn, where it represented 20% of the

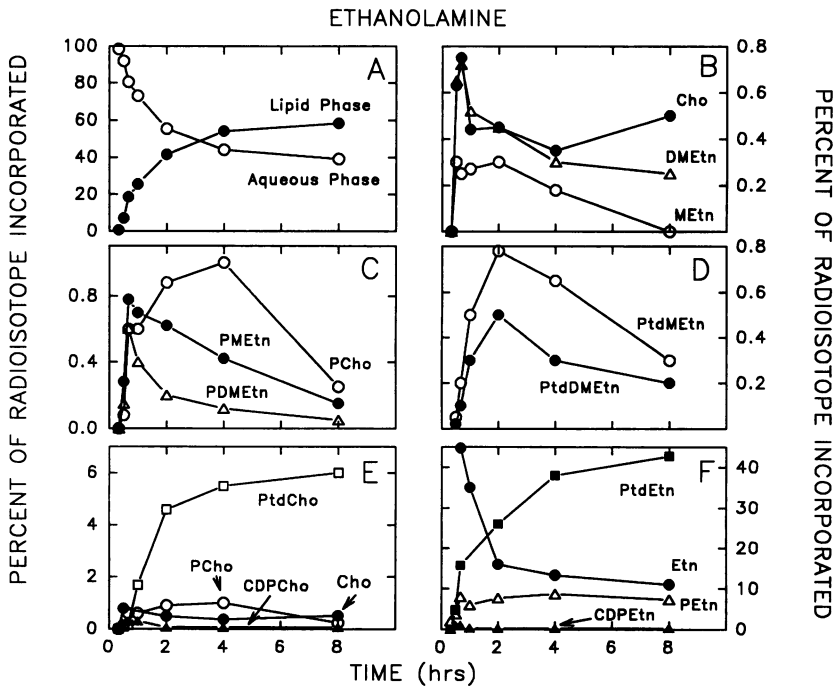

Figure 2. Radioactive products resulting from feeding castor bean endosperm a 10-min pulse with [2-^{14}C]Etn followed by a chase with non-radioactive Etn (80 nmol/endosperm half).

total radioactivity incorporated into the endosperm (Fig. 3F). A small amount of label was also detected in CDP-Etn (Fig. 3F). The amount of radioactivity in PtdEtn increased with time as label was lost from Etn, P-Etn, and CDP-Etn.

At the end of the pulse, label was also detected in MEtn, DMEtn, and Cho (Fig. 3B), as well as P-MEtn, P-DMEtn, and P-Cho (Fig. 3C) but not in PtdCho, PtdMEtn, or PtdDMEtn (Fig. 3D). The incorporation of radioactivity into DMEtn paralleled the increase and decrease in MEtn, which suggests that DMEtn was synthesized by methylation of MEtn (Fig. 3B). The incorporation of radioactivity into P-DMEtn preceded the appearance of label in P-MEtn, but at 30 and 60 min after the pulse, the labeling in P-MEtn was twice that of P-DMEtn (Fig. 3C). Therefore, P-DMEtn may have two origins, P-MEtn by methylation and DMEtn by phosphorylation. After 1 h, the amount of radioactivity in these two phospho-bases, P-MEtn and P-DMEtn, decreased with time as label was accumulated into P-Cho (Fig. 3C), PtdMEtn, and PtdDMEtn (Fig. 3D). The radiolabel in PtdMEtn and PtdDMEtn could be accounted for by a methylation pathway at the phosphatidyl level or by incorporation of P-MEtn and P-DMEtn, respectively, by the appropriate cytidylyltransferase and base phosphotransferase. The radioactivity did not appear in CDP-Cho until after 60 min, even though PtdCho was already labeled (Fig. 3E). This suggests another source of label for PtdCho other than the nucleotide pathway, such as the phosphatidyl methylation pathway. After the appearance of significant levels of ^{14}C in Cho, between 120 and 240 min after the pulse, there was no change in the labeling pattern of PtdCho. Thus, externally added P-Etn does not seem to be the source of Cho for the CDP-Cho pathway, but it does seem to be the source of P-Cho for the CDP-Cho pathway. Again, the initial reaction would be the methylation of free Etn, however.

Metabolism of [^{14}C]P-Ser

P-Ser, subjected to paper chromatography with solvent B, migrated together with P-Etn derivatives. When the peak was eluted and chromatographed with solvent C, it separated into three peaks in the first half of the sheet representing P-MEtn, P-DMEtn, and P-Cho, plus a single peak not far from the front, which contained P-Ser. Ser, in solvent B, migrated alone and more slowly than did P-Etn.

After a 10-min pulse with [^{14}C]P-Ser, with a purity of 88%, 65% of the total radioactivity incorporated into the endosperm appeared in Ser, whereas the amount of label remaining in P-Ser was very low (Fig. 4A). At this time, only the free bases Etn, MEtn, and DMEtn were labeled (Fig. 4B). During the first 30 min of the chase period, the amount of radioactivity increased in MEtn, DMEtn, and Cho as label was lost from P-Ser, Ser, and Etn. Thereafter, the loss of label from Etn, MEtn, and DMEtn appeared to coincide with the increased label in Cho (Fig. 4B), P-Etn, P-MEtn, and P-DMEtn (Fig. 4C). This suggests that Etn was synthesized by decarboxylation of Ser, Cho was synthesized by methylation of Etn, and P-MEtn and P-DMEtn had two origins: (a) from the corresponding free base by a kinase reaction and (b) from P-Etn by methylation.

The amount of radioactivity incorporated into PtdEtn and PtdCho increased with time (Fig. 4D). Externally added P-Ser was incorporated into PtdSer, PtdMEtn, and PtdDMEtn at a much lower rate. Whether PtdEtn was synthesized via PtdSer decarboxylation, Etn base exchange, or CDP-Etn is unclear, but the latter seems the most likely (Fig. 4F).

The parallel labeling of PtdEtn and PtdCho suggests that some of the labeled PtdCho was synthesized by methylation of labeled PtdEtn (Fig. 4D). The labeling in P-Cho and in CDP-Cho suggests that part of the labeled PtdCho was also

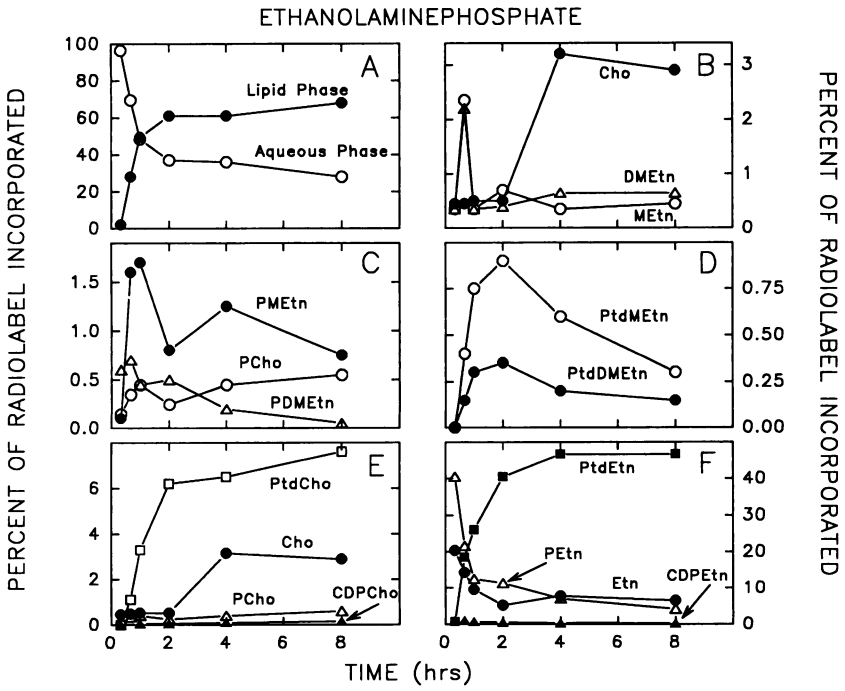

Figure 3. Radioactive products resulting from feeding castor bean endosperm a 10-min pulse with [^{14}C]P-Etn.

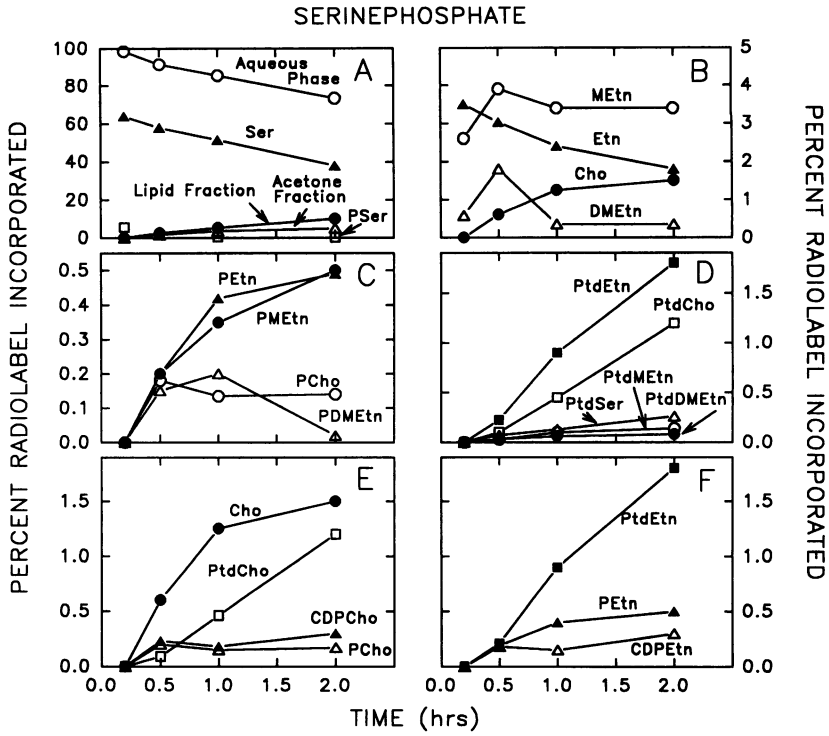

Figure 4. Radioactive products resulting from feeding castor bean endosperm a 10-min pulse with [^{14}C]P-Ser.

synthesized via the nucleotide pathway (Fig. 4E). But whether the free Cho produced is a source of PtdCho is unclear. Cho could either accumulate in a pool that is inaccessible for PtdCho synthesis, similar to free Ser, or be the source of PtdCho through the CDP-Cho pathway (Fig. 4E). This latter possibility cannot be ruled out.

Metabolism of [*methyl*-^{14}C]Cho

Endosperm halves were labeled continuously for 1 h in the presence of 4.5 nmol of Cho containing radioactive methyl groups. Radioactivity accumulated in P-Cho (22.8% of total radioactivity incorporated) and in PtdCho (22.5%), whereas only traces were detected in CDP-Cho (0.7%).

DISCUSSION

In recent publications, Mudd and Datko (6, 23) concluded that, in *Lemna*, soybean, and carrot, the first methylation leading to PtdCho synthesis occurred at P-Etn, because this compound was rapidly methylated and little or no radioactivity was detected in Etn derivative bases. They reported the presence of an enzyme capable of converting P-Etn to P-MEtn in the three plant systems investigated but found no reaction with free Etn bases (7). These results led them to postulate that in some, and perhaps all, higher plants the methylation of P-Etn is the common committing step in PtdCho synthesis. The data reported here provide evidence that castor bean endosperm does not conform to this hypothesis, because this methylation seems to utilize Etn, and not P-Etn, as the initial substrate, thereby forming MEtn rather than P-MEtn as the initial product. Indeed, MEtn became rapidly and relatively highly labeled whether the precursor administered was AdoMet, Etn, P-Etn, or P-Ser. The possibility may be excluded that the radioactivity observed in MEtn arose by artifactual breakdown of P-MEtn during extraction, because, under the conditions used, the recovery of [*methyl*-^{14}C]P-Cho was high, with only 2 to 5% of the total radioactivity being found in Cho following overnight chromatography in solvent C. Therefore, in castor bean endosperm, we suggest placing the initial step at the methylation of free Etn. What is not clear, however, is how directly this leads to synthesis of PtdCho in this tissue.

Immediately after the [^{14}C]P-Etn pulse, Etn was the major labeled aqueous product. This result cannot be attributed to an unspecific, nonenzymic degradation of the precursor, because P-Etn is relatively stable and its purity tested right before the experiment was 92 and 94%. Therefore, it seems likely that this Etn was released from P-Etn by a phosphatase; the specificity of this phosphatase remains unknown. Another possibility is that Etn was released from P-Etn by a reversal of Etn kinase, in a manner similar to that proposed for Cho kinase (14). P-Etn for the nucleotide pathway could arise either directly from the exogenously applied radiolabel or from P-Etn synthesized from Etn provided by the phosphatase reaction followed by an Etn kinase-catalyzed reaction. It seems unlikely that PtdEtn was the product of only an Etn base exchange reaction, which would produce radiolabeled phospholipid by exchange of free Etn for a preexisting headgroup (29), because this reaction is less than 1% as active as the nucleotide pathway when activities of base exchange (29) and Etn phosphotransferase (31) are compared.

When [^{14}C]P-Ser was the administered precursor, Ser was the major labeled aqueous compound after the pulse. In *Lemna*, even after a short incubation time of 5 min with

[^{14}C]P-Ser, Ser was the only labeled compound in the aqueous fraction (24). This result is not due to a nonenzymic breakdown of P-Ser, because P-Ser is relatively stable and was 88% radiopure before the experiment, but it is possible that this Ser was the product of a phosphatase. This theory also was put forth by Mudd and Datko (24), but the pathway appears unrelated to what has been thought to be the normal in vivo metabolism of P-Ser (16, 27, 30). This may be a result of passing through the cytosol, which might be an unusual compartment for this intermediate. On the other hand, the reaction may have been catalyzed by phosphoserine phosphatase, an enzyme of the so-called phosphorylated pathway of Ser synthesis, which, in nonphotosynthetic plant tissues such as seeds, meristems, roots, and nodules, is associated with plastids and thought to be the primary pathway of Ser synthesis (16, 27, 30).

In *Lemna*, a number of experiments provided no support for P-Ser being the direct precursor of P-Etn, but the results are not in conflict with the possibility of a small, rapidly turning over pool of P-Ser that is accessible to Ser and, therefore, to the phosphoserine phosphatase (24). Our own results included P-Etn as one of several products, but it is unclear how directly it arises, because free Etn appears before P-Etn (Fig. 4, B and C).

The fate of [^{14}C]P-Ser was different from that of L-[^{14}C]Ser previously reported for this same tissue (13). The main differences between the utilization of exogenously added Ser and Ser derived internally from externally added P-Ser are: (a) their relative importance to PtdSer, PtdEtn, and PtdCho synthesis and (b) the relative kinetics of labeling of the nucleotide pathway components Cho, P-Cho, CDP-Cho, and PtdCho. Kinney and Moore (13) noted that exogenously added Ser entered PtdSer and PtdEtn, and thereby PtdCho, through the methylation pathway, but did not appear to arrive at PtdCho via the nucleotide pathway; the Cho, P-Cho, and CDP-Etn formed from the added Ser seemed to be inaccessible to the enzyme catalyzing PtdCho synthesis, possibly as a result of compartmentalization. The results of our experiments indicate that Ser produced endogenously from administered P-Ser leads to P-Cho and CDP-Cho, which are utilized for PtdCho synthesis, therefore appearing to enter a different pool from that of administered free Ser. However, we have no evidence yet as to whether the results reflect a true difference between endogenously produced Ser and exogenously added Ser, or whether the two groups of data are not comparable because the techniques used were different. The monomethyl and dimethyl intermediates were not examined in the L-[^{14}C]Ser study (13). Ser is subject to a number of additional reactions, of course, and this was emphasized by the finding that under similar conditions phospholipids became labeled not only in their base moieties but also in their diacylglycerol portions, as well as the methyl groups of their methylated Etn-containing bases (24). This would, of course, complicate interpretation of long-term studies of the phospholipid synthesis from this precursor.

Results of the pulse-chase experiments with P-Etn and P-Ser would also support the methylation of PtdEtn as another source of label for PtdCho in addition to the nucleotide pathway. The source of PtdEtn could be by decarboxylation of Ser to Etn and thereby through P-Etn and CDP-Etn, by decarboxylation of PtdSer (13) and/or by an Etn base exchange reaction (13, 29). The amounts of radioactivity detected in PtdMEtn and PtdDMEtn were always extremely small compared with PtdEtn and PtdCho, suggesting that their pool size also is small relative to that of PtdEtn and PtdCho. Moore (19) suggested that PtdCho synthesis by the methylation pathway involving only phosphatidyl derivatives was unlikely to be the major one in castor bean endosperm. This suggestion is supported by the in vitro enzyme activities of each pathway, which are about 10% as active as the nucleotide pathways (19), and the in vivo rates of incorporation of [^{14}C]AdoMet and [^{14}C]Cho into PtdCho (13).

The potential substrate leading to methylated PtdEtn is uncertain because for each precursor studied both MEtn and PtdMEtn became rapidly labeled. It is, therefore, impossible to conclude whether PtdMEtn is formed from MEtn through a nucleotide (CDP-requiring) pathway or by methylation of PtdEtn or by both routes. It is of interest that not only Moore (19), utilizing castor bean endosperm, but also Marshall and Kates (18) with spinach leaves and Datko and Mudd (7) with *Lemna*, soybean, and carrot cultures could not demonstrate the in vitro methylation of PtdEtn, whereas they were able to detect an AdoMet methylation of PtdMEtn and PtdDMEtn. Furthermore, Wang (unpublished data) demonstrated that cytidylyltransferase can utilize either P-MEtn or P-Cho equally to form the CDP derivatives, and Moore (20) observed that under exchange reaction conditions MEtn was incorporated, and the resulting PtdMEtn was successively methylated to form PtdDMEtn and PtdCho. Under the same conditions, neither L-Ser nor Etn, which are known to undergo exchange reactions (29), yielded PtdCho. Despite this, the methylation of PtdEtn to PtdMEtn, which is a dominant pathway in animals and microorganisms (32), cannot be completely ruled out for the castor bean endosperm or, perhaps, for plant systems in general. Indeed, it remains possible that the AdoMet:PtdEtn methyltransferase was inactivated during cell breakage as suggested by Marshall and Kates (18) or that the first methylation step is rate limiting as postulated by Moore (19).

Exogenously added Cho is immediately available for PtdCho synthesis. However, as also mentioned in the introduction, the source of free Cho for PtdCho synthesis in the cells is unknown. Externally added L-Ser seems not to be such a precursor in castor bean endosperm. AdoMet, Etn, and P-Etn kinetics of incorporation into Cho did not seem to correlate with changes in PtdCho labeling and, therefore, appeared to be independent of PtdCho synthesis. Thus, although these compounds support the synthesis of Cho, this Cho seems not to be available for PtdCho synthesis. A test of the effect of hemicholinium-3, a Cho kinase inhibitor, on the incorporation of these radiolabeled substrates into PtdCho (13) provided results that support this conclusion. Hemicholinium-3 decreased added Cho incorporation into PtdCho by 65%, whereas it reduced the incorporation of Etn or P-Etn utilization for PtdCho synthesis by only 15 and 42%, respectively, and produced no change at all in AdoMet incorporation. With P-Ser, the quantity of Cho synthesized was always higher compared with P-Cho, CDP-Cho, and PtdCho, and therefore, only a portion of the Cho, if any at all, was involved in PtdCho synthesis.

One possible model compatible with the results reported here is that the castor bean endosperm possesses (a) a sequestered pool of Cho in which the bulk of the Cho synthesized from externally added L-Ser, Etn, AdoMet, P-Etn, and P-Ser accumulates and (b) a smaller metabolic pool of Cho in which exogenously administered Cho and part of the Cho synthesized from the L-Ser obtained by dephosphorylation of P-Ser enters and is available to Cho kinase. Thus, Ser, Etn, P-Etn, and probably P-Ser are unlikely to be the direct sources of Cho for the CDP-Cho pathway in castor bean endosperm. But is the free Cho required for PtdCho synthesis? Kinney and Moore (14) showed that at physiological pH the reverse reaction of Cho kinase is favored and that its in vitro activity was constant during the development of the endosperm, as opposed to the other two enzymes of the nucleotide pathway and to PtdCho content and PtdCho synthesis (15). Therefore, Cho may actually represent an intermediate on a pathway that is moving away from PtdCho. It seems more likely that P-Cho would be the first committed molecule of the CDP-pathway, as appears to be the case for *Lemna* and carrot cultures (6), and its synthesis requires methylations that might occur at both the free base and the phosphorylated base levels, the first step being the methylation of Etn and with phosphorylation occurring before the formation of choline (Fig. 5). Another source that also must be considered for this tissue is recycling from preexisting PtdCho, as has been noted for cotton cotyledons (3).

These results lead us to propose the scheme shown in Figure 5 as a working model. The initial methylation is placed at Etn, which itself is synthesized by decarboxylation of Ser. More direct in vitro evidence for the existence of such a reaction, and its basic requirements, are described in the following paper. The resulting MEtn is converted partially to P-MEtn, and the remainder is methylated to DMEtn. The DMEtn also is partitioned between methylation to Cho and phosphorylation to P-DMEtn. This latter compound may also be the product of P-MEtn methylation and appears to be the major substrate for P-Cho synthesis. P-Cho is finally incorporated into PtdCho through the nucleotide pathway. The larger amount of radioactivity found in P-Cho compared with CDP-Cho supports the hypothesis that the rate-limiting step in the nucleotide pathway of PtdCho synthesis is at the cytidylytransferase reaction. This conclusion is also supported by work concerning the kinetic characteristics of the cytidylyltransferase isolated from castor bean endosperm (15, 33). The same assumption may be put forward for the regulation of PtdEtn synthesis through the CDP-pathway, because CDP-Etn accumulated much less radioactivity than P-Etn. Thus, the rate limiting step in PtdEtn synthesis would be at the cytidylytransferase and not at the ethanolamine kinase reaction. However, no attempt at purification of P-Etn cytidylyltransferase and elucidation of its role in regulation of PtdEtn synthesis has been reported for plant systems.

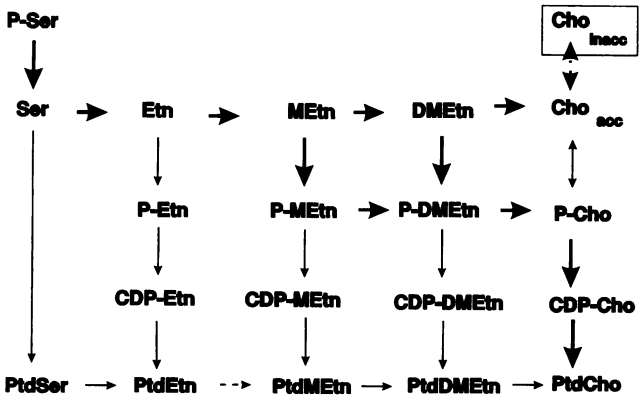

Figure 5. Tentative working model of the pathways for synthesis of PtdCho in castor bean endosperm. Heavy arrows, Major fluxes; solid-line arrows, significant fluxes; dashed arrows, minor fluxes; dotted arrow, transport and/or equilibration. Abbreviations: Cho_{acc}, Cho accessible to the PtdCho synthesis pathway; Cho_{inacc}, Cho inaccessible to the PtdCho synthesis pathway.

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