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Phosphatidylcholine Synthesis in Castor Bean Endosperm[1]

I. METABOLISM OF L-SERINE

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ABSTRACT

Endosperm halves from 3-day-old castor bean (*Ricinus communis* var Hale) were incubated for 30 minutes with L-[^{14}C]serine, after which label was observed in ethanolamine, choline, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, ethanolaminephosphate, and CDPethanolamine, but not in cholinephosphate or CDPcholine. Only later did significant amounts of isotope become incorporated into cholinephosphate and CDPcholine. The choline kinase inhibitor hemicholinium-3 prevented the incorporation of label from serine into cholinephosphate and CDPcholine, reduced the incorporation of [^{14}C]choline into phosphatidylcholine by 65%, but inhibited the incorporation of label into phosphatidylcholine from serine by only 15%. The inhibitor did not prevent the incorporation of labeled methyl groups from *S*-adenosyl-L-methionine into phosphatidyldimethylethanolamine plus phosphatidylcholine. The amount of incorporation of label from the methyl donor was only 8% of that from choline into phosphatidylcholine. The implications of these results for the pathway and regulation of phosphatidylcholine synthesis from the water-soluble precursors are discussed.

Phosphatidylcholine is the major phospholipid in most eukaryotic cell membranes, with phosphatidylethanolamine generally being second most abundant (1). Some evidence exists for regulation of the levels of PC[2] independent of the other phospholipids in plants. For example, PC increases as a proportion of the total phospholipid over the 8-d postgermination period in castor bean endosperm (13, 16). This regulation may be complex, however, since PC synthesis involves more than one pathway (10, 12). For instance, in an early study of the incorporation of [^{14}C]serine into intact tomato roots, Willemot and Boll (18) concluded that PE was synthesized by the decarboxylation of PS, and PC by the methylation of PE. This pathway also has been found in castor bean endosperm (10), but the activity appeared to be only a fraction of the activity of the enzymes of the nucleotide pathway, in which PC is synthesized from free choline. Thus, at least two pathways must be considered as sources for PC in plants.

The source of the water-soluble precursors also is unclear. It seems likely that in many plant tissues the biosynthesis of choline involves the methylation of ethanolamine, and that ethanolamine is formed by the decarboxylation of serine (6). It is not firmly established, however, whether these reactions occur predominantly at the level of free bases, phosphoryl bases, or phosphatides. In other words, it is not clear if the syntheses involve only the water soluble substrates or a complex interrelationship between cytosolic and membrane-bound intermediates. Studies in some plant tissues, utilizing both precursor incorporation (8, 14) and enzyme studies (9, 10), appear to indicate that the methylation reaction occurs mainly at the phosphatide level. On the other hand, in spinach (5) and sugar beet leaves (7) the methylation reaction exists at the phosphoryl base level and not at all at the phospholipid level. Furthermore, there appears to be very little known about the serine decarboxylation reaction in any plant tissue. Although in some mammalian tissues it is clear that PS is a major precursor of PE (17), studies in plant tissues have been complicated due to apparently high turnover rates (18), low levels of PS (11), or an apparent lack of incorporation of radiolabeled precursors into the PS fraction (2). Marshall and Kates (9) reported the presence of a PS decarboxylase enzyme in spinach leaves, but its localization in a cytoplasmic fraction raises the possibility that the *in vivo* enzyme may also be involved in the decarboxylation of free serine. This possibility was not tested. Serine decarboxylases have not, to our knowledge, been examined in plant tissues.

To continue our studies (10, 13) of the regulation of synthesis of PC in plants, and to assess the relative importance of the aqueous and lipid precursor pathways in the synthesis of PE and PC, we have examined the fate of [^{14}C]serine applied to castor bean endosperm as well as the effect of two inhibitors of phospholipid synthesis on the incorporation of various headgroup precursors into PS, PE, and PC.

MATERIALS AND METHODS

Growth of Plants. Seeds of castor bean (*Ricinus communis* L. var Hale) were removed from their seedcoats, surface sterilized with 10% bleach solution, and planted, under sterile conditions, in vermiculite moistened with sterile water. They were germinated in the dark for 3 d at 30°C.

Serine and Inhibitor Treatment. Estimation of serine utilization was done by incubating endosperm halves treated on the abaxial surface with 10 μl of ^{14}C-L-serine (0.2 μCi/endosperm half) for 30 min and then washing with distilled H_2O prior to further incubation. For the inhibitor studies the endosperm from each plant was separated into two halves; one half was incubated for 10 min with 10 μl of either 50 mM diethylethanolamine or 50 mM hemicholinium-3 applied as a drop to the abaxial surface; the second half was incubated with an equal quantity of deionized H_2O. Thirty endosperm halves from 15 plants were used for each experiment. After a 10 min incubation the halves were washed with deionized H_2O and then treated for 60 min with radiolabeled precursor (0.2 μCi/endosperm half). After the incubation periods the endosperm halves were rinsed in distilled H_2O and extracted as described below.

Extraction and Chromatography. At the specified intervals washed endosperm halves were frozen in liquid N_2 and ground to a fine powder in a mortar and pestle. The powder was extracted

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[2] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SAM, *S*-adenosyl-L-methionine.

with chloroform-methanol (2:1, v/v) for 2 h at −20°C. The organic and aqueous phases were separated (3) and analyzed by chromatography.

Lipids in the chloroform fraction were separated (15) on 250 μM silica gel G thin layer plates in a solvent system consisting of chloroform:2-propanol:triethylamine:methanol:0.25% KCl (w/v) (30:25:18:9:6, v/v).

Better TLC separation of the aqueous intermediates was obtained if the extract was first passed through an ion-exchange column. For this the aqueous-methanol fraction was evaporated to dryness and redissolved in 20 mM Hepes buffer (pH 7.0). This was applied to an 0.8 × 4 cm ion-exchange column packed with AG1-X8 (200–400 mesh) ion exchange resin in the formate form and equilibrated with 20 mM glycylglycine buffer (pH 9.0). The column retained 70% of the free bases and more than 95% of the phosphoryl and nucleotide bases; the mechanism of this retention is not clear. If the column was equilibrated to pH 7.0 with Hepes buffer, instead of pH 9.0, only the nucleotide bases were retained. Labeled compounds bound to the pH 9.0 column were eluted with 2 ml of 0.1 M NH_4HCO_3. After the NH_4HCO_3 elution, less than 1% of the free and phosphoryl and 20% of the nucleotide bases remained bound to the column. Labeled compounds in both the first, Hepes, elution, and in the NH_4HCO_3 elution were separated by TLC on 500 μM silica gel G thin layer plates using methanol:0.6% KCl (w/v):NH_4OH (50:50:5, v/v) in the first dimension and, after drying the plate in air, 1-butanol:propionic acid:water (2:1:1.3, v/v) in the second dimension.

Detection and Counting. Labeled lipids and other compounds were detected by spraying the thin layer plates with ENHANCE (New England Nuclear) and developing at −80°C with Kodak autoradiography film for 2 d.

To measure radioactivity, labeled compounds were eluted from the silica with methanol, which then was evaporated to dryness in scintillation vials. The sample radioactivity was measured in Aquasol (New England Nuclear) using a Beckman LS-8000 scintillation counter.

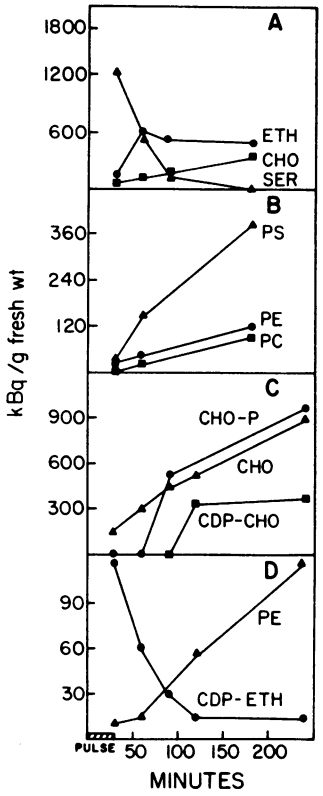

FIG. 1. Radioactive products resulting from feeding castor bean endosperm a 30 min pulse with ^{14}C-L-serine. The different panels represent data from separate experiments. Abbreviations are: (A) CHO, choline; ETH, ethanolamine; SER, serine; (B) PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; (C) CDPcho, CDPcholine; CHO, choline; CHO-P, cholinephosphate; (D) CDPeth, CDPethanolamine; PE, phosphatidylethanolamine.

RESULTS AND DISCUSSION

Radioactivity from [^{14}C]serine appeared in both ethanolamine and choline at the end of a 30 min labeling period (Fig. 1A). At this time label also was detected in CDPethanolamine, PS, PE, and PC, but not in cholinephosphate or CDPcholine (Fig. 1, A–C). A small amount of label also was detected in ethanolaminephosphate (about 150 cpm/g fresh weight) at 30 min, but disappeared within 60 min and was not observed at any other time, pointing to a strong demand on that substrate. There was some variability in the uptake of serine between experiments, but the relative distribution of the total, incorporated label among the metabolites of the serine did not vary (results not shown).

The amount of radioactivity in PS increased with time (Fig. 1B), as label was lost from serine (Fig. 1A), and accounted for about 35% of the original label in serine after 3 h. Some of the increased label in PE appeared to coincide with the loss of label from CDPethanolamine (Fig. 1D) although the bulk of the label in PE appeared after 90 min when all the label in CDPethanolamine had already been depleted (Fig. 1D). This suggests another source of label for the PE, such as PS. The incorporation of radioactivity into PC preceded the appearance of label in cholinephosphate or CDPcholine and appeared to follow and parallel the increase in PE (Fig. 1B). This implies that some of the labeled PC was synthesized by methylation of labeled PE.

The quantity of phosphatidylcholine synthesized from serine via the CDPcholine pathway appears to be low. Aqueous ethanolamine appeared to be rapidly methylated to free choline, and this in turn was converted to cholinephosphate and then CDPcholine (Fig. 1, A and C). However, the radioactivity did not appear in CDPcholine until 90 min, even though PC was labeled by 30 min. This occurred despite the fact that label incorporation into cholinephosphate and CDPcholine was 10 times greater than the radioactivity incorporated into PC. There was no change in the labeling pattern of PC even after the appearance of significant levels of ^{14}C in CDPcholine. By way of contrast, exogenously administered choline is immediately and readily available for PC synthesis (12; Table I). These results raise the distinct possibility that choline produced from externally added serine is only minimally, if at all, available for PC synthesis. Externally added ethanolamine was incorporated into PC at a very low rate, but this may have been through the PE methylation pathway (Table I).

The inhibitor studies support these conclusions. Hemicholinium-3, a choline kinase inhibitor (4), reduced the incorporation of exogenous serine into PC by only 15%, whereas it reduced added choline incorporation into PC by 65% (Table I). Furthermore, after a 10 min incubation with hemicholinium-3 and a 180 min incubation with [^{14}C]serine, no label could be observed in either cholinephosphate or CDPcholine, whereas the amount of label in PC was almost the same as the control with distilled water in place of the inhibitor (results not shown). These data support the proposition that the exclusive pathway for synthesis of PC from externally added serine is through the methylation pathway.

The incorporation of ^{14}C-SAM into phosphatidyldimethylethanolamine (PDME) + PC occurred at about 8% of the rate of incorporation of choline into PC after 60 min, even though the precursors were taken up by the tissue to a similar extent. This

Table I. *Effects of Diethylethanolamine (DEE) and Hemicholinium-3 (HC3) on the Incorporation of Several Radiolabeled Substrates into Phospholipids*

The results are the means of 3 experiments (duplicates in each) and with a standard deviation less than 10% of the mean. Recoveries (total counts from the TLC plates *versus* counts in the extract) were approximately 70%.

Substrate	Phosphatidylserine			Phosphatidylethanolamine			Phosphatidylcholine		
	Control	DEE	HC3	Control	DEE	HC3	Control	DEE	HC3
	kBq/g fresh wt	*% of control*		*kBq/g fresh wt*	*% of control*		*kBq/g fresh wt*	*% of control*	
Serine	183	29	85	179	21	85	69	19	85
Ethanolamine				1547	35	59	172	120	85
Ethanolamine-P				1528	19	51	43	28	58
Choline							4758	8	34
Choline-P							694	14	60
SAM							365	149	114

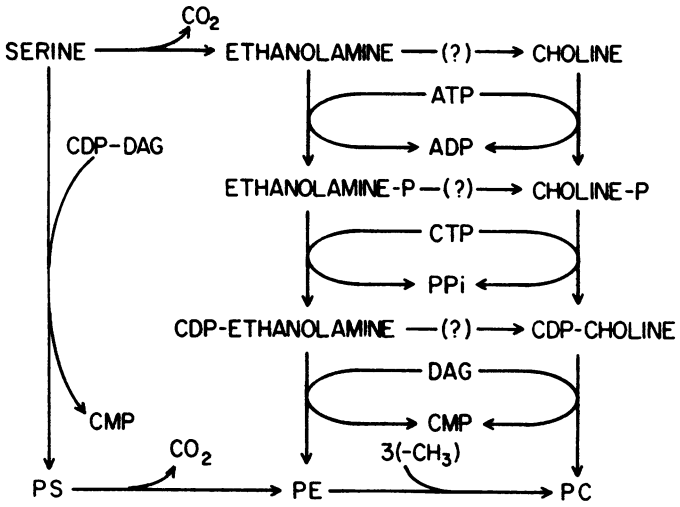

FIG. 2. An outline of potential pathways for syntheses of phosphatidylcholine from serine. The data support the incorporation of [^{14}C] from serine into PC by way of PE methylation. The source of PE could be by decarboxylation of PS, serine to ethanolamine and thereby through CDPethanolamine, or by both pathways. Although introduced serine does support the synthesis of choline, cholinephosphate and CDPcholine, these do not appear to be available for synthesis of PC.

is in agreement with the previous suggestion, based on the relative enzyme activities of the nucleotide and methylation pathways, that the methylation pathway was about 10% as active as the nucleotide pathway (10). It is apparent, therefore, that although label from [^{14}C]serine appeared to be incorporated into PC entirely by methylation of PE during the experiment, the nucleotide pathway has the capacity for being the major pathway of PC synthesis in this tissue.

Incorporation of SAM into PDME + PC was not inhibited by hemicholinium-3 (Table I). No label was observed in phosphatidylmonomethylethanolamine (PMME) although some labeling was observed in an unidentified lipid which co-chromatographed with the neutral lipids on the TLC plates. Hemicholinium-3 did inhibit the incorporation of ethanolamine into PE by 40%, in comparison with only a 15% inhibition of the serine to PE conversion. These data support an exclusive incorporation of methyl groups from SAM via the PE methylation pathway and not by methylation of aqueous ethanolamine. They also allow the occurrence of some decarboxylation of PS or ethanolamine base exchange to form PE, thus leaving these reactions as potential sources for the PE to be methylated to PC.

Diethylethanolamine inhibited the incorporation of all the labeled precursors into lipids by 70 to 90%, with the exceptions of ethanolamine into PC (which appeared to be slightly stimulated) and [^{14}C]SAM (which was stimulated by about 50%). Since both ethanolaminephosphate and choline incorporation into PC were severely inhibited by diethylethanolamine, the appearance of label in PC from [^{14}C]ethanolamine during diethylethanolamine treatment (in some experiments not observed at all) probably was due to base exchange (11).

In summary, these data indicate that the route of externally added serine into PC in castor bean endosperm is by the PE methylation pathway (Fig. 2). Whether the PE for this purpose is synthesized via CDPethanolamine, ethanolamine base exchange or PS decarboxylation is unclear, but the latter two seem most likely. Free choline produced from the serine appears unavailable for major incorporations into PC via choline base exchange or the nucleotide pathway, despite the fact that the potential for the nucleotide pathway is much higher than for methylation. To explain these data, we propose a working model with two compartments, one in which externally added serine can be utilized for PS and PE synthesis, and thereby ultimately to PC, and another in which cholinephosphate and CDPcholine can be formed but which are isolated from the final enzyme in PC synthesis, CDPcholine:diacylglycerol cholinephosphotransferase. The identity of these compartments and the source of choline for the CDPcholine pathway remain to be determined.

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