Phosphatidylserine Synthesis in Castor Bean Endosperm

THOMAS S. MOORE, JR.
Department of Botany, University of Wyoming, Laramie, Wyoming 82071

ABSTRACT
Phosphatidylserine synthesis by the endoplasmic reticulum fraction isolated from castor bean (Ricinus communis var. Hale) endosperm was assayed by measuring the incorporation of \(^{14}\)C-L-serine into chloroform-soluble material. Both phosphatidylserine and phosphatidylethanolamine were identified as products. The incorporation required calcium ions and showed an optimum pH of 7.8 in 2 mM CaCl\(_2\). Phosphatidylethanolamine and CDP-diglyceride stimulated the reaction only about 40 to 50% and primary alcohols had relatively little effect on the incorporation. These and other results suggest the synthesis of phosphatidylserine in this tissue occurs by an exchange reaction but the relative roles of phospholipase D and phosphatidylethanolamine:L-serine phosphatidytransferase remain to be elucidated.

Evidence to date shows that the synthesis of phosphatidylserine can occur by either of two pathways. These can be summarized as:

\[
\begin{align*}
\text{Phosphatidylethanolamine} + \text{L-serine} & \rightarrow \text{phosphatidylserine} + \text{ethanolamine} \\
\text{CDP-diglyceride} + \text{L-serine} & \rightarrow \text{phosphatidylserine} + \text{CMP}
\end{align*}
\]

(1) (2)

The first pathway, known as the exchange reaction, seems to be the exclusive means of phosphatidylserine synthesis in animal tissue (9, 21) and Tetrahymena (6). \(\text{Ca}^{2+}\) is an absolute requirement for this reaction. This route of synthesis is largely microsomal with some mitochondrial activity in mammalian tissue (9, 21), but probably only mitochondrial in Tetrahymena (6). Pathway 2 is characteristic of E. coli (9, 19, 21) and has no \(\text{Ca}^{2+}\) requirement.

Phosphatidylserine is a known constituent of plant membranes, but little work has been performed to characterize its mode of synthesis in plants (11, 16). Although it is usually present in small quantities, it may play a very significant role in those membranes; efforts to understand the metabolism of this phospholipid should aid in understanding that role. Early efforts by Willemot and Boll (23) suggested that \(^{14}\)C-serine served as a precursor of phosphatidylserine, with subsequent decarboxylation forming phosphatidylethanolamine in excised tomato roots, but further characterization of this system was not attempted. Vander and Richard-

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Materials and Methods

Materials. The salts were from J. T. Baker Company. Phosphatidylethanolamine (plant) and phosphatidylserine (bovine) were purchased from Miles Laboratories, and the CDP-diglyceride (synthetic dicoley) was from Serdary Research Laboratories (London, Ontario, Canada). All other organic compounds except sucrose (from Mallinkrodt) were obtained from Sigma. \(\text{L}-\text{Serine-1-}\text{\textsuperscript{14}C}\) was purchased from ICN and \(\text{L-serine-3-}\text{\textsuperscript{14}C}\) was used from both ICN and Amersham/Searle Corp.

Because of a chloroform-soluble impurity (about 0.1% of the total counts) found in \(\text{\textsuperscript{14}C}-3\)-serine from both sources, this isotope was dissolved in water, washed four times with chloroform, and the water phase was dried under a stream of nitrogen. It was then resuspended in water to give the proper concentration.

Plant Material. Seeds of castor bean (Ricinus communis var. Hale) were soaked in running tap water for 1 day and germinated in moist vermiculite covered with aluminum foil at 30 C.

Homogenization. This procedure was similar to methods described previously (18). Thirty endosperm halves, removed from 4 to 4.5-day-old seedlings, were chopped for 15 min with a single razor blade in 11 ml of grinding medium contained in a Petri dish on ice. The grinding medium contained 150 mM Tricine (pH 7.5), 10 mM KCl, 1 mM MgCl\(_2\), 1 mM EDTA (pH 7.5), and 15% (w/w) sucrose.

Fractionation of Cellular Components. The crude homogenate was filtered through two layers of Dacron cloth and the remaining debris was removed by centrifuging at 270g for 10 min. Five milliliters of the supernatant were layered on gradients composed of: (a) a 6-ml cushion of 60% (w/w) sucrose; (b) 20 ml of sucrose solution decreasing linearly in concentration from 60 to 32% (w/w) sucrose; and (c) a 5-ml layer of 20% (w/w) sucrose, containing in a 37.5 ml tube. All sucrose was prepared in 1 mM EDTA (pH 7.5).

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The gradients were centrifuged at 20,000 rpm for 4 hr in a Beckman Model L5-50 preparative ultracentrifuge equipped with a Spinco Model SW-27 rotor and maintained at 4°C. Separation into serial 0.6-ml fractions and monitoring of the absorbance at 280 nm was accomplished with an Isco Model 640 fractionator equipped with a UA-5 absorbance monitor. Fractions corresponding to the endoplasmic reticulum (13, 14) were pooled.

Enzyme Assay. The method for measuring the incorporation of 14C-serine into the chloroform-soluble fraction was derived from the assay of Raghavan et al. (20). The reaction usually was run for 1 hr at 30°C in a final volume of 0.5 ml containing 40 mM HEPES buffer (pH 7.8), 2 mM CaCl2, and 0.4 mM 14C-L- or 14C-3-serine (0.5 μCi; chloroform-washed 14C-3-serine was used). The reaction was started by the addition of 100 to 150 μg of endoplasmic reticulum fraction.

The reaction was stopped and extracted according to the method of Bligh and Dyer (3). Stopping the reaction by the addition of ethanol (80%, v, v, final concentration) followed by extraction by the method of Folch et al. (8) gave similar results. The radioactivity of the chloroform fraction was determined with a Beckman LS-100 scintillation counter in a scintillation cocktail consisting of 6 g of PPO and 100 g of naphthalene in 1000 ml of dioxane. Corrections were made with an internal standard.

Chromatography. Identification of the products was accompanied by TLC co-chromatography with known standards on Silica Gel G in three solvent systems (1, 7, 10): (a) chloroform-methanol-acetic acid-water (170:25:25:6, v, v), (b) chloroform-methanol-water (65:25:4, v, v); and (c) chloroform-methanol-ammonium hydroxide (65:25:4, v, v).

RESULTS

The general requirements for the incorporation of L-serine into chloroform-soluble products are given in Table I. Boiling the endoplasmic reticulum fraction led to an 88% loss of activity and the omission of Ca2+ an absolute requirement of the mammalian exchange reaction, led to similar results. The preferred optical isomer of serine is L-serine since 1 mM D-serine does not compete with the incorporation of the 14C-L-serine; the addition of 1 mM L-serine inhibits isotope incorporation by 54%. Two other compounds which are known to participate actively in exchange reactions (9, 21), choline and ethanolamine, gave 4% and 68% inhibition of L-serine incorporation, respectively, indicating ethanolamine can be used in the reaction but choline is used to only a very limited extent. Preliminary tests showed the reaction is essentially linear for 2 hr and over 200 μg of protein.

The addition of either phosphatidylethanolamine or CDP-diglyceride to the reaction mixture led to similar results (Table II). Both lipids promoted the reaction about 40 to 50% at the highest concentrations used. Because both lipids gave similar data, it is unlikely that the response is attributable to a specific substrate action, but probably to disruption of the membrane structure leading to improved access of the enzyme to the serine. Triton X-100, however, inhibited the reaction at concentrations as low as 0.025%.

Two distinct slopes of increasing enzyme activity were found with increasing L-serine concentration and saturation of the reaction was not obtained at concentrations as high as 400 μM (Fig. 1). Whether this biphasic nature of the graph is attributable to the presence of two enzymes catalyzing the reaction, to changes in the enzyme environment at the higher serine concentrations, or to some other phenomenon, cannot be explained at this time. The maximum rates obtained in these studies are quite low, but the concentration of phosphatidylserine in the membranes also is very low (7).

The requirement of the enzyme for calcium is satisfied over a broad concentration range, with concentrations of 2 to 5 mM giving maximal activity at pH 7.8 (Fig. 2). A more distinct optimum at 2 mM CaCl2 was obtained with 9 μM L-serine rather than 400 μM. It should be noted that the reaction mixture contains approximately 0.2 mM EDTA because endoplasmic reticulum

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<th>Table II. Effects of Adding Various Concentrations of Phosphatidylethanolamine and CDP-diglyceride to the Assay Mixture</th>
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![Fig. 1](#)

**FIG. 1.** Effects of increasing L-serine concentration on serine incorporation into the chloroform-soluble fractions.
fractions were used directly from the density gradients. Mg++, Mn++, Cu++, Co++, and Zn++ did not substitute for Ca++. One study of the exchange reaction in rat liver has demonstrated a dramatic shift in the pH optimum from 7.5 to over 9 when the Ca++ concentration is increased from 0.16 mM to 4 mM (2). Such a shift did not occur here (Fig. 3) although a slight shift to a lower pH optimum of around pH 7 did occur with increasing Ca++ concentration in most experiments. A pH optimum of around 7.8 was obtained using 2 mM Ca++. Although HEPES is a poor buffer at the two limits tested, it maintained the pH at the lower limit and drifted only slightly at pH 9.

There are two final chloroform-soluble products of the reaction identifiable by TLC. They are phosphatidyserine, as expected, and phosphatidylethanolamine. Approximately 48% of the product was found in phosphatidyserine and 52% in phosphatidylethanolamine. Phosphatidylethanolamine can be derived directly from phosphatidyserine by the decarboxylation of the serine moiety (9, 21); the addition of 14C-1-serine in place of 14C-3-serine results in a 49% apparent reduction in the incorporation and only one radioactive spot co-chromatographing with phosphatidyserine, lending support to the synthesis of phosphatidylethanolamine here being due to this decarboxylation.

Phospholipase D, which has been suggested to be the enzyme responsible for the exchange reaction (9), can transfer the phosphatidyl unit to certain primary alcohols instead of the natural phospholipid acceptor in the presence of high concentrations of these alcohols (4, 5, 24). In the present case, in which the concentrations of the alcohols used were those described by Vandor and Richardson (22), ethanol inhibited the incorporation of L-serine 35%, whereas methanol inhibited 10% and glycerol had no effect.

**DISCUSSION**

The data presented in this paper support the exchange reaction as the pathway for synthesis of phosphatidylserine in the castor bean endosperm. A basic cation requirement for this reaction, Ca++, has been demonstrated. The absence of a distinct or specific response to either phosphatidylethanolamine or CDP-diglyceride also may indicate that the exchange reaction is the probable mode of synthesis because phosphatidylethanolamine is the second most abundant phospholipid in this membrane. Phosphatidylethanolamine constitutes approximately 18.8% of the membrane phospholipid (7), and so may occur in high concentrations at the enzyme site and thus be readily available for the exchange reaction. On the other hand, the addition of CDP-diglyceride is required for the synthesis of phosphatidylglycerol in this tissue (17) and so should be rate limiting for the synthesis of phosphatidylserine if it also were a precursor for this reaction. The ability of ethanolamine, but not choline, to inhibit L-serine incorporation implies that phosphatidylethanolamine rather than phosphatidylcholine is the phospholipid substrate. Definitive evidence must await isolation of the enzyme.

The apparent exchange for serine in the castor bean resembles the exchange for ethanolamine described by Vandor and Richardson (22) in that Ca++ is required at similar concentrations. The present system, however, seemed to be more stable under the reaction conditions, giving a linear increase for up to 2 hr whereas theirs leveled off after 45 min. The pH optimum is also slightly different, theirs being at pH 8.5 instead of pH 7.8. In addition, they found that D-serine and choline inhibited ethanolamine incorporation which is not true for the L-serine incorporation reported here. Whether these differences simply reflect the different tissues or materials used or reflect some fundamental difference between the utilization of ethanolamine as opposed to serine is unknown.

The enzyme described here does not strongly resemble phospholipase D described in other systems. That phospholipase reportedly is found either in plastids or the soluble fraction of the cell, requires high Ca++ concentrations, and has an acid pH optimum (12), whereas the enzyme in castor beans is found exclusively in the endoplasmic reticulum (18), requires less Ca++, and needs a more neutral pH. Also, phospholipase D reportedly utilizes serine in an exchange reaction to only a slight extent (12). In addition, Vandor and Richardson (22) found that their membrane-bound exchange reaction was not active with alcohols as is phospholipase D. The enzyme studied here, while showing different susceptibilities to alcohols than that of Vandor and Richardson, still did not show inhibition of L-serine incorporation as extensively as would be expected with phospholipase D (4, 5, 24). On the other hand, the above evidence is indirect and the biphasic nature of the increase in enzyme activity with increasing L-serine concentration (Fig. 1) might be interpreted as two enzymes...
involved in the synthesis. Further studies are underway to measure directly and characterize the various phospholipases in the castor bean endosperm during germination.

The immediate decarboxylation of about half of the incorporated serine to form phosphatidylethanolamine agrees with the data of Vandor and Richardson (22), suggesting this decarboxylation occurs in plant microsomes. On the other hand, the decarboxylation activity has been found exclusively in the mitochondria of rat livers (9, 21), and both synthesis and decarboxylation activities seem to occur in the mitochondria of *Tetrahymena* (6). The role of this decarboxylation in plant tissues is obscure. It is unlikely that the major synthesis of phosphatidylethanolamine is catalyzed by this reaction, in light of the small amount of phosphatidylserine synthesis measured in this tissue and the findings of Macher and Mudd (15) that ethanolamine incorporation can occur in plants using CDP-ethanolamine as a precursor.

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LITERATURE CITED