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PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN CASTOR BEAN ENDOSPERM. MECHANISMS OF REGULATION DURING THE IMMEDIATE POSTGERMINATION PERIOD*

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Key Word Index—Castor bean; phosphatidylcholine; biosynthesis.

Abstract—Changes in phosphatidylcholine content, lipid precursor incorporation and lipid biosynthetic enzyme activities were observed in castor bean endosperms during a six day postgermination period. The metabolism of phosphatidylcholine was regulated independently of other phospholipids, as evidenced by the ratio of phosphatidylcholine to total phospholipid not being constant during development. The main pathway for phosphatidylcholine biosynthesis was the nucleotide pathway, although studies with labelled glycerol-3-phosphate suggested that the diacylglycerol for this pathway was not synthesized *de novo*, but probably obtained by degradation of pre-existing lipids. The control of the nucleotide pathway appears to reside in choline phosphate cytidylyltransferase and choline phosphotransferase, the activities of which were coordinated, but not in choline kinase. The cytidylyltransferase is regulated in part by translocation of the soluble form of the enzyme to the endoplasmic reticulum, but this regulation could not account for all of the observed changes in phosphatidylcholine synthesis.

INTRODUCTION

Stored triacylglycerols are broken down and converted into sucrose in the endosperm during the immediate postgermination period of castor bean [1, 2]. The majority, if not all, of the fatty-acid oxidation occurs in the glyoxysomes [3]. These organelles are rapidly produced in the young endosperm and function during the first five days after germination [4,5] As the endosperm matures, and the triacylglycerol is depleted, the glyoxysomes are reduced in number [4]. As phosphatidylcholine (PC) is the major membrane phospholipid of these organelles [6, 7], it might be expected that there would be large changes in its metabolism, corresponding with synthesis of glyoxysomes, during the early stages of endosperm development. Indeed, the PC content of endosperm has been shown to change rapidly during the first few days postgermination [8], but the contribution of *de novo* PC synthesis to these changes is not clear

Changes in the *in vitro* activity of cholinephosphotransferase, the final enzyme of the nucleotide pathway for PC biosynthesis [9], have been observed during endosperm development. These changes did not completely correlate with the changes in PC content, however [8]. Changes in the rate of PC synthesis from choline were not reported in the previous study, nor were the activities of the two other enzymes of the nucleotide pathway, choline kinase [10] and choline phosphate cytidylyltransferase [11] The possibility of variations in the contribution of phosphatidylethanolamine methylation [9] to PC synthesis during development also have not been reported, although in three-day-old endosperm this pathway is thought to contribute less than 10% of the total PC synthesis [12,13].

In this study, we report changes in the incorporation rates of radiolabelled precursors of the nucleotide pathway of PC synthesis and relate them to changes in *in vitro* enzyme activity and *in vivo* PC content.

RESULTS

Lipid content

As our conditions for germination and growth are different from those utilized for previous studies of this nature utilizing castor bean endosperm [1,2,8], we feel it necessary to provide basic information on growth for comparative purposes. Under our conditions the fresh weight increased to day six, while dry weight of endosperm decreased *ca* 40% (Fig. 1) The total lipid content decreased from about 550 mg/30 halves to 25 mg/30 halves during this six day period (Fig. 2A). The total phospholipid content remained relatively constant at *ca* 10 mg/30 halves (Fig. 2B) throughout the six days, with only a small increase between days one and two (Fig. 2B) Phosphatidylcholine content initially increased between days one and two, from 3 to 3.5 mg/30 halves, and then decreased to 1.25 mg/30 halves by day six (Fig. 2C).

Precursor uptake and incorporation

All of the precursors applied to the endosperm were taken up and utilized, with uptake by endosperm halves

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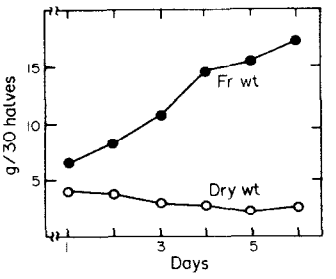

Fig. 1 Fresh and dry weights of castor bean endosperm germinated and grown as described in the Experimental

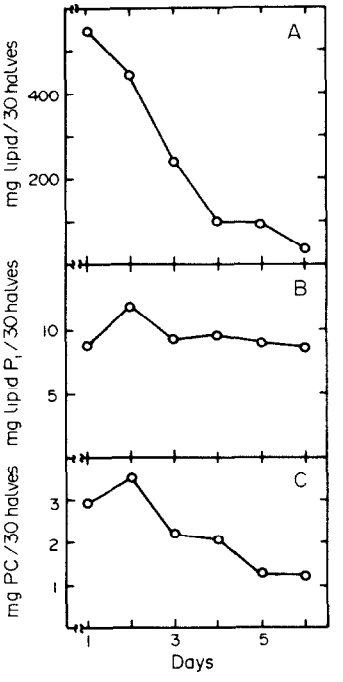

Fig. 2 Total lipid (A), lipid phosphorous (B) and phosphatidylcholine (C, separated by TLC and quantitated by a phosphorous assay, see Experimental) content of castor bean endosperm during a six day postgermination period

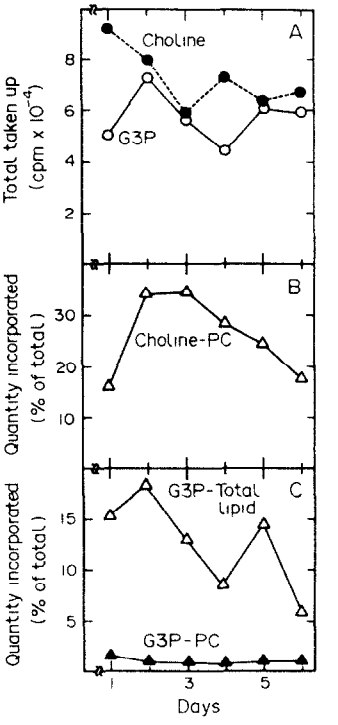

Fig 3 The (A) amount of radiolabelled choline (●—●) and glycerol 3-phosphate (○--○) taken up by endosperm tissue in 30 min at different ages post-imbibition, (B) the per cent of this radiolabel incorporated into phosphatidylcholine for choline (as well as choline-P and CDPcholine), and (C) total lipid or phosphatidylcholine labeled by glycerol-3-phosphate The radiolabels were applied on the abaxial surface as described in the Experimental Abbreviations Cho, choline, Cho-P, phosphocholine, CDP-Cho, CDPcholine, G3P, glycerol-3-phosphate

of different ages being fairly similar. The average uptake rates for choline and glycerol-3-P were 2500 cpm/min and 2000 cpm/min, respectively (Fig 3A). The rate of incorporation of choline into PC increased between days one and two with 35% of the incorporated choline appearing in PC at days two to three. This was followed by a reduction to 5–17% of the label taken up during the remaining days. Similar results were obtained for choline-P and CDPcholine Most of the radiolabel (6 to 18% of the label taken up) from glycerol-3-phosphate (G3P) which was incorporated into lipid (Fig. 3C) appeared in phosphatidic acid. A constant 2% of the label taken up appeared in PC.

Enzyme activities

The protein contents of the 100 000 *g* supernatant, band A and ER from 30 endosperm halves over the 6 day post-imbibition period are shown in Fig 4. The total protein of the supernatants (Fig 4A) increased between days 1 and 2, and then decreased through day 5. The protein content of band A (Fig 4B) increased between days 1 and 2 and then decreased until day 4, after which time it remained constant ER protein increased from day 1 until day 3 and then decreased between days 3 and 4, after which time it remained constant (Fig. 4B).

The choline kinase activity of 100 000 *g* supernatants was fairly constant from 1 to 6-day old endosperm tissue (Fig. 5A). More than 95% of the choline kinase activity of the total cell homogenate was recovered in the supernatants from tissue of all ages examined, and further extraction of the tissue residue resulting from the original homogenization of 1- to 6-day-old endosperm, by grinding the residue with 10 ml of homogenization medium in a mortar and pestle for 10 min, resulted in no additional choline kinase activity. It therefore was assumed that the relative recovery of total enzyme activities from tissues of different ages was similar and essentially complete.

Cytidylyltransferase (both band A and ER; Fig. 5B) and choline phosphotransferase (in the ER, Fig 5E) increased from day 1 until day 3 and thereafter decreased The soluble cytidylyltransferase activity decreased from 1.45 nmol/min/30 halves at day 1 to no detectable activity at day 6 (Fig. 5C) The ratio of ER to supernatant activity, however, increased from day 1 to day 3, and then

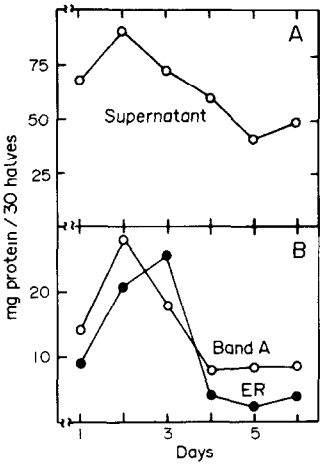

Fig. 4 Protein content of the supernatant (A) and the ER and Band A (B) of castor bean endosperm at different times post-imbibition.

decreased through day 6. The supernatant activity in endosperm up to 3-days-old could be stimulated *ca* three-fold by the addition of a total lipid extract from 3-day-old endosperm (Fig 5C). The lipid extract was first evapd to dryness and then suspended by sonication in 100 mM Hepes buffer (pH 7 0) to a final phospholipid concentration of 0 4 mM, as determined by lipid phosphorous. The highest activity measured in lipid-treated supernatants was 3 31 nmol/min/30 halves (Fig 5C) at day one, and it remained near this level through day three. Lipid stimulation of soluble cytidylyltransferase from day four or older endosperm tissue was considerably less (Fig. 5C).

DISCUSSION

The decrease in total lipid content during the development of the endosperm probably corresponds to the breakdown of storage fats and is in general agreement with previous studies [1, 2]. Marriott and Northcote [2] have shown that the phospholipid content of endosperm increases slightly during the first six days after germination in contrast to the constant amounts observed in this study after day two (Fig. 2B) Their results were expressed on a dry weight basis, however, and the dry weight of the endosperm decreases slightly during this time period (Fig. 1) This may account for the small increase they reported

The overall profile of PC content in this study was similar to that of Moore and Troyer [8], except that in the previous study the peak was obtained at day 4 while in this study the peak was at day 2. This discrepancy probably arises from the conditions under which the seeds were germinated. In the previous study the seeds were germinated with the seed coats intact, following overnight imbibition, while the current study utilizes seeds planted immediately after mechanical removal of the seed coats This latter method results in faster, more consistent rates of growth with germination percentages of 80–90%. The PC content of the endosperm appears to vary independently of the bulk phospholipid. For example, the small change observed in total phospholipid content between days one and three can be accounted for by changes in the PC content of the

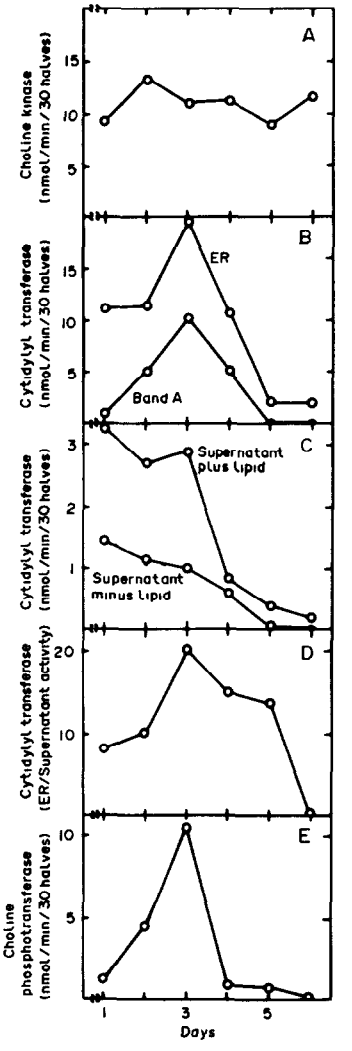

Fig. 5. Activities of choline kinase (A), cytidylyltransferase of the ER and Band A (B), cytidylyltransferase of the supernatant in the presence or absence of endosperm total lipid extract (C), and cholinephosphotransferase in the ER (E) of castor bean endosperm at various times post-imbibition Panel D presents the ratios of ER-bound cytidylyltransferase activity to that of the supernatant at various times postgermination.

endosperm. After day three, the PC content (Fig. 2C) decreased, unlike the total phospholipid (Fig. 2B). This may represent the rise and fall of specific organelle membranes. For example, glyoxysomal membranes are known to contain high concentrations of PC [6, 7] and enzymes of that organelle increase in activity early in the postgermination period, followed by a later decrease [1]. On the other hand, other membranes relatively lower in PC (mitochondria, plastid) may be more stable. The changes in incorporation of choline paralleled these changes in PC content. These data suggest that the PC composition of these cells was determined mainly by changes in PC synthesis and that PC breakdown was constant over the six-day period.

About 20% of the label from G3P was incorporated into phospholipid, most of it into phosphatidic acid, with only a very small, constant amount of this label being

detected in PC. The incorporation of G3P label into PC did not coincide with either the changes in PC content or in the incorporation of nucleotide pathway precursors Since G3P is the precursor of diacylglycerol [13], which reacts with CDPcholine to form PC [13], it seems likely that most of the diacylglycerol used for PC synthesis in this tissue was not synthesized *de novo* from G3P but utilized a lipid precursor from some other source, possibly from recycling of other lipids.

The *in vitro* activities of cytidylyltransferase and choline phosphotransferase increased sharply between days one and three and then decreased in parallel (Fig 5B, E), suggesting that the activities of these two enzymes are tightly coupled. This profile correlates well with the rate of PC synthesis in this tissue. By way of contrast, the *in vitro* activity of choline kinase did not correlate with the changes in PC content, PC synthesis, or the activities of the other two enzymes of the pathway (Fig 5) It appears, then, that this enzyme is not involved in regulation of PC biosynthesis during the development of castor bean endosperm. Thus, the most likely enzymes for controlling the nucleotide pathway are the membrane-bound cytidylyltransferase and cholinephosphotransferase.

The changes in activity of cytidylyltransferase in band A were similar to the changes in ER activity (Fig 5B) This observation supports our previous suggestion that this fraction is an artifact of the homogenization procedure [11] and probably represents a subfraction of the ER

The cytidylyltransferase activity in the soluble fraction did not appear correlated with the membrane cytidylyltransferase or with changes in PC synthesis (Fig 5C) We have previously shown that the ER cytidylyltransferase from three-day-old endosperm could be solubilized with 0 2 M potassium chloride, and that the solubilized enzyme activity was strongly promoted by PC and oleate [11] This led to the suggestion that the cytidylyltransferase in this tissue may be regulated by translocation of the soluble enzyme to ER membranes [11], a mechanism previously described for mammalian tissue [14] The results presented here further support this suggestion since the ratio of ER to soluble activity doubled from days one to three (Fig 5D), and a three-fold stimulation of soluble activity was observed in the presence of castor bean phospholipids (Fig. 5C) during days one through three The magnitudes of change in the soluble activity, however, were not sufficient to account for all of the changes in the membrane activity. Maximum stimulation of the soluble enzyme from one-day-old endosperm in the presence of lipids resulted in an activity of *ca* 3.3 nmol/min/30 halves (Fig 5C), whereas the ER activity increased from 11 nmol/min/30 halves to 20 nmol/min/30 halves from days one to three (Fig 5B) It seems likely that there are additional mechanisms which regulate the activity of this enzyme

The coordination of membrane cytidylyltransferase with cholinephosphotransferase activity during the first six days of endosperm development, and the correlation of these increases with increases in PC content of the tissue, lend support for these enzymes playing a role in regulation of the synthesis of this phospholipid It is uncertain at this time whether the changes in activities of these enzymes are caused only by changes in the amounts of enzyme protein or by direct regulation of the enzyme activities. A definitive answer must await purification of the proteins involved Such purifications are particularly difficult for these membrane-bound proteins whose activity is rapidly lost following solubilization Nonetheless, data such as these do allow a definition of which enzymes to concentrate on, what materials to use, and potential mechanisms of regulation

EXPERIMENTAL

Plant material Castor bean seeds were germinated and grown as previously described [11]

Lipid extraction and analysis 30 endosperm halves of the required age were frozen in liquid N_2 and ground to a fine powder with a mortar and pestle Lipids were extracted with $CHCl_3$–MeOH and separated by TLC as previously described [12, 15] Total lipid content of the extracts was determined gravimetrically Phospholipids were eluted from the TLC plates and lipid phosphorus determined as described in ref [15]

Precursor incorporation Endosperm halves were incubated with [Me-^{14}C]-labelled choline (0 42 μCi/half), choline phosphate (0 17 μCi/half), CDPcholine (0 04 μCi/half) or [U-^{14}C]-labelled L-glycerol-3-phosphate (0 33 μCi/half) The specific compounds were first diluted in 100 mM Hepes buffer (pH 7 0) and 100 μl of this labelled soln were applied evenly to the abaxial surface of each endosperm half The endosperm halves were incubated for 30 min in disposable petri dishes (5 halves/dish) after which time they were washed for 2 min in distilled H_2O and then frozen with liquid N_2 The frozen tissue was ground into a fine powder and extracted as previously described [12] Total precursor uptake was estimated as the total amount of radiolabel incorporated into the lipid fraction, aq fraction and post-extraction residue of the washed tissue Incorporation of label into phospholipids was estimated as described in ref [16] and was expressed as a percentage of the total label incorporated into the tissue All experiments were repeated 3 times, the standard error of replication was 5% or less

Homogenization and centrifugation 30 endosperm halves were chopped (2 strokes/second) with a single razorblade for 10 min at 4° and pH 7 5 in 150 mM Tricine containing 500 mM sucrose, 10 mM KCl and 3 mM EDTA For preparation of soluble fractions, the homogenate was filtered through 2 layers of cheesecloth and centrifuged at 250 g_{av} for 10 min The resultant supernatant was centrifuged at 100 000 g_{av} for 60 min in a Sorvall OTD-65B ultracentrifuge using a Sorvall T875 rotor Endoplasmic reticulum (ER) and 'band A' membranes were isolated on sucrose density gradients as previously described [11]

Enzyme assays Choline kinase, membrane-bound choline phosphate cytidylyltransferase and cholinephosphotransferase were assayed as previously described [10, 11, 13] Soluble cytidylyltransferase was assayed in a medium containing a higher specific activity of ^{14}C-cholinephosphate (0 5 Ci/mol) than the standard assay medium [11] used for the ER and band A enzyme. The final incubation medium for the soluble cytidylyltransferase consisted of 10 mM [^{14}C]-choline phosphate (0 5 Ci/mol), 2mM CTP, 10 mM $MgCl_2$ and 25 μl of supernatant in a final vol of 50 ul of 100 mM Hepes buffer (pH 7 0) All other details of the assay were identical to that for membrane cytidylyltransferase [11] Protein was determined by the method of ref [16]

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