

Rapid Degradation and Limited Synthesis of Phospholipids in the Cotyledons of Mung Bean Seedlings¹

Received for publication December 26, 1978 and in revised form March 2, 1979

NEIL R. GILKES, ELIOT M. HERMAN, AND MAARTEN J. CHRISPEELS²

Department of Biology, C-016, University of California/San Diego, La Jolla, California 92093

ABSTRACT

Seedling growth of mung bean is accompanied by the rapid catabolism of the three major phospholipids in the cotyledons (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol). The decline starts 24 hours after the beginning of imbibition and by the 4th day of growth more than 50% of the phospholipids have been catabolized. Extracts of cotyledons of 24-hour-imbibed beans contain enzymes capable of degrading membrane-associated phospholipids *in vitro*. This degradation involves phospholipase D and phosphatase activity.

Studies with radioactive acetate, glycerol, and orthophosphate indicate that the three major phospholipids are also synthesized in the cotyledons. Incorporation of glycerol and acetate into phospholipids of cotyledons is relatively constant throughout seedling growth, while the incorporation of [³²P]orthophosphate steadily declines from a high value 24 hours after the start of imbibition. The newly synthesized phospholipids become associated with membranous organelles, especially the endoplasmic reticulum, and have an *in situ* half-life of 2 to 2.5 days.

Determination of the activities of two enzymes involved in phospholipid biosynthesis (phosphorylcholine-glyceride transferase and CDP-diglyceride-inositol transferase) shows that the enzymes have their highest activities 12 hours after the start of imbibition. High activities for both enzymes were found in cotyledons of beans incubated at 1 C, indicating that the enzymes may preexist in the dry seeds.

The experiments demonstrate that cotyledons start synthesizing new phospholipids immediately after imbibition, but that the rate of phospholipid catabolism far exceeds the rate of synthesis long before the cotyledons start to senesce.

Germination and seedling growth of seeds are accompanied by the mobilization of the reserves present in the storage tissues (cotyledons, endosperm, etc.). These reserves are usually contained in special organelles such as protein bodies, amyloplasts, or fat droplets. The mobilization process may require the synthesis of new enzymes and the transport of these enzymes to the storage organelles, or even the biogenesis of new organelles responsible for the breakdown of reserves. There is increasing evidence that the ER plays an important role in the biosynthesis of enzymes which need to be transported intracellularly, and in the biogenesis of cytoplasmic organelles (for a review on ER in plant cells, see ref. 4).

Our work on the mobilization of protein reserves in mung bean cotyledons has led us to investigate the role of the ER in this process. Mobilization is dependent on the biosynthesis of a new

endopeptidase which is synthesized in the cytoplasm and transported to the protein bodies (2). The synthesis and/or transport of the enzyme may be mediated by the ER (5). We are investigating the morphology of the cisternal and tubular ER in the cotyledons, as well as biochemical changes in ER function. Because the ER is the major site of phospholipid synthesis (19) we have investigated the biosynthesis, degradation, and turnover of phospholipids in the cotyledons. The results indicate that the cotyledons are capable of phospholipid biosynthesis very soon after imbibition, but that phospholipid catabolism exceeds synthesis, resulting in a rapid net decline in phospholipid content. This decline precedes the catabolism of reserve proteins and the senescence of the cotyledons.

MATERIALS AND METHODS

Growth Conditions. Seeds of mung bean (*Vigna radiata* L. Wilczek) were germinated in the dark as previously described (6). In one experiment the axes were removed from dry seeds and cotyledons incubated on moist sterile sand (14).

Extraction, Separation, and Quantitation of Phospholipids. Lipids were extracted from tissue with either chloroform-methanol (10), or hot isopropyl alcohol.

In the first method, samples were homogenized in a mortar in 5 ml 50 mM Tris-HCl (pH 7.5) at 0 C, followed by centrifugation at 850g for 3 min to remove cell wall debris and starch. The supernatant was then extracted with 2 volumes of chloroform-methanol (2:1, v/v). The organic phase was removed, washed twice with 2 volumes of methanol-water (1:1, v/v) containing 0.8% NaCl, and evaporated to dryness.

In the second method samples were homogenized in 2 ml hot isopropyl alcohol and centrifuged at 2,000g 5 min. The pellet was reextracted with 2 ml chloroform-isopropyl alcohol (2:1, v/v), centrifuged, and the supernatants pooled and evaporated to dryness.

Lipid extracts were redissolved in chloroform-methanol (2:1, v/v), and applied to TLC plates (New England Nuclear, Silica Gel Type 0, 250 μ m thickness). Development was in petroleum ether-acetone (1:3 v/v), followed by chloroform-methanol-acetic acid-water (50:25:7:3, v/v/v/v) in the same dimension. Identification of lipids was by co-chromatography of reference compounds after visualization with iodine vapor.

Phosphate in lipid extracts or individual phospholipids from thin layer chromatograms was assayed after digestion in 72% perchloric acid by the method of Bartlett (1).

Assay of Phospholipid-degrading Enzymes. Cotyledons were homogenized in 100 mM Tris-HCl (pH 7.5) and the 27,000g, 20-min supernatant was used as a source of enzyme after the removal of the fat layer. An aliquot of enzyme (50 μ l) was incubated with 50 μ l of ³²P-labeled membranes or ³²P-labeled PC³ in 1 ml of buffer (K-acetate, 50 mM, pH 5.3) containing 40 mM CaCl₂. The

¹ This research has been supported by a grant from the National Science Foundation (Metabolic Biology).

² To whom inquiries should be addressed.

³ Abbreviations: PC: phosphatidylcholine; PE: phosphatidylethanolamine; PA: phosphatidic acid; PI: phosphatidylinositol.

reaction was stopped by the addition of 4 ml of chloroform-methanol (2:1, v/v), the lipids in the organic phase were recovered and fractionated by TLC. The radioactivity in the aqueous phase was also recovered and partitioned into organic and inorganic [^{32}P]phosphate according to Saha and Good (20).

Incubations with [^{32}P]PC were done with 1 μmol of PC (200,000 cpm) and 1.5 mg of Nonidet P-40 to make lipid micelles. Incubations with membranes were done with 2 μmol of membrane-associated phospholipid (300,000 cpm). ^{32}P -labeled membranes were obtained by germinating 100 beans for 18 h in 1 mCi of [^{32}P]Pi and homogenizing the beans in 30 ml of 100 mM Tris-HCl (pH 7.5). The cleared homogenate (5 min at 1,000 rpm) was loaded on a discontinuous sucrose gradient (20% sucrose and 35% sucrose in 100 mM Tris-HCl, pH 7.5) and centrifuged for 1 h at 82,000g. The membranes at the 20/35 interface were used as a source of ^{32}P -labeled membranes.

Assays of Phospholipid-synthesizing Enzymes. Cotyledons were homogenized in 10% (w/w) sucrose, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM MgCl_2 and the 2,000g, 5-min supernatant layered on a discontinuous gradient consisting of 20% over 35% (w/w) sucrose in the above medium. Gradients were centrifuged at 95,000g for 90 min and particulate material at the 20/35 interface was collected. This microsomal fraction was used as a source of enzyme.

Phosphorylcholine-glyceride transferase was assayed as described by Johnson and Kende (13). The reaction mixture contained in 0.3 ml: 150 mM Tris-HCl (pH 7.0), 30 mM MgCl_2 , and 0.125 μCi [methyl- ^{14}C]cytidine diphosphocholine, 49 Ci/mol. The reaction was initiated by addition of 50 μl membrane suspension (equivalent to 2.5 cotyledons), and incubated for 30 min at 30 C.

CDP diglyceride-inositol transferase was assayed by the method of Sumida and Mudd (21). The reaction mixture contained in 0.45 ml: 100 mM Tris-HCl (pH 8.0), 2 mM MnCl_2 , 2 mM cytidine triphosphate, and 10 μCi *myo*-[2- ^3H (N)]inositol, 17.4 Ci/mmol. The reaction was initiated by addition of 50 μl membrane suspension and incubated for 30 min at 30 C.

Incorporation of Radioactive Phospholipid Precursors. Groups of 10 cotyledons were sliced (six to seven slices per cotyledon), rinsed in water and incubated in 2 ml water containing 2 mg chloramphenicol and labeled precursor (3.6 μCi [1,3- ^{14}C]glycerol, 15.76 Ci/mol; 7.0 μCi [1- ^{14}C]acetic acid, 58.3 Ci/mol; or 53 μCi [^{32}P]Pi carrier-free; all purchased from New England Nuclear Corp.). The tissue was then rinsed with a 1% solution of the appropriate unlabeled precursor and lipids extracted with isopropyl alcohol or chloroform-methanol as described above. Precursor uptake was determined by counting an aliquot of the aqueous homogenate supernatant. In [^{32}P]Pi labeling experiments incorporation into the organic phosphate fraction was determined by the method of Saha and Good (20). Incorporation into individual phospholipids was measured after separation by TLC.

Turnover Experiments. Groups of 15 intact seedlings were labeled by submersion of the cotyledons in 2 ml of water containing 2.3 μCi [1,3- ^{14}C]glycerol (15.8 mCi/mmol), for 2.5 h at room temperature. Seedlings were then rinsed in 1% glycerol; a control group of 15 seedlings was removed at this point for determination of incorporation at zero time, and the other seedlings were planted in moist Vermiculite. Groups of 15 seedlings were removed at 1-day intervals and the incorporation of [^{14}C]glycerol in the phospholipids determined.

Sucrose Gradients. Cotyledons from 3-day-old seedlings were sliced and incubated with [^{14}C]glycerol for 2.5 h (5 μCi /8 cotyledons). The tissue was homogenized in 2.2 ml of 50 mM Tris-HCl (pH 7.5), 0.1 mM MgCl_2 , 1 mM EDTA, 10% (w/w) sucrose, and the 2,000g, 3-min supernatant layered on a 12 to 50% (w/w) sucrose gradient in the same medium. After centrifugation at 160,000g for 16 h the gradient was fractionated (0.45-ml fractions) and radioactivity determined by collecting a trichloroacetic acid precipitate on a membrane filter (type HAWP 025 00 Millipore

Corp.). An aliquot of each fraction was used to assay NADH Cyt *c* reductase by following the change in A_{550} . The reaction mixture contained 80 mM Tris-HCl (pH 7.5), 0.2 mM KCN, 0.5 mg NADH, and 0.8 mg oxidized Cyt *c*.

RESULTS

Phospholipid Content of Cotyledons. Two different methods were used to extract phospholipids from the cotyledons prior to the determination of phospholipid phosphorus in the individual phospholipids. In the first method an aqueous homogenate of the cotyledons was extracted with chloroform-methanol (2:1) while in the second one the cotyledons were homogenized directly in boiling isopropyl alcohol. The second method extracted 1.2 to 1.5 times more PC and PE and up to 10 times more PI than the first method. The second method was therefore preferred for quantitative determination. Cotyledons of dry seeds contained about 200 nmol of phospholipid phosphate per cotyledon and 90% of it could be accounted for by the three major phospholipids: PC (50%), PE (30%), PI (9%). The amounts of the minor phospholipids were not determined. PA accounted for less than 3% of the total phospholipid phosphate at all stages of seedling growth indicating that little or no breakdown of phospholipids resulting from the action of phospholipase D occurred during the extraction process.

Changes in the amounts of PE, PC, and PI in the cotyledons as a function of time of germination and seedling growth are shown in Figure 1. PE and PC declined steadily starting 12 h after the beginning of imbibition, while the PI content doubled during the first 24 h and declined subsequently. Half the phospholipid phosphate was lost during the first 4 days of growth.

Separation of the axis from the cotyledons followed by the incubation of isolated cotyledons on moist sand resulted in a considerable slow down in the rate of catabolism of the phospholipids. When the cotyledons remained attached to the plant 66% of the phospholipid phosphate was lost between the 1st and the 5th days of growth (Fig. 1). When the isolated cotyledons were incubated on moist sand this rate of loss slowed to less than half, and only 26% of the phospholipids were lost during this time period. The decline in PC, PE, and PI occurred in parallel as in the control plants (data not shown).

Phospholipid-degrading Enzymes. The rapid catabolism of the phospholipids *in vivo* indicates that the cotyledons must have enzymes capable of degrading these molecules. The presence of some of these enzymes can be demonstrated by incubating ^{32}P -

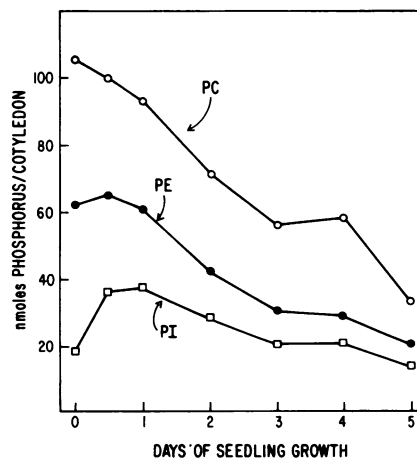


FIG. 1. Levels of major phospholipids in mung bean cotyledons during seedling growth. Lipids were extracted from fresh cotyledons with hot isopropyl alcohol. Phospholipids were separated by TLC and the amount of Pi in each phospholipid determined after digestion with hot HClO_4 . Amounts of minor phospholipids were not determined. PA never accounted for more than a few % of the total.

labeled membranes with an aliquot of cotyledon homogenate at pH 5.3 in the presence of 40 mM CaCl₂ and following the course of phospholipid degradation. Such an experiment (Fig. 2A) showed that PC and PE were rapidly degraded, while PA accumulated. These data indicate that PC and PE are converted to PA. This conversion is known to be catalyzed by phospholipase D. PA itself was much more stable and only 10% of the ³²P-labeled PA was lost during the subsequent 4-h incubation. Incubation of ³²P-labeled PC in PC-Nonidet micelles with an aliquot of a cotyledon extract also resulted in the rapid degradation of PC and the accumulation of PA (Fig. 2B). In addition we observed a slow release of water-soluble ³²P-labeled compounds equivalent to 10% of the PC at the end of the 90-min incubation period. Fractionation of these ³²P-labeled compounds into organic and inorganic phosphate by the method of Saha and Good (20) showed that 85% of the radioactivity was in inorganic phosphate. The release of inorganic phosphate from PA is known to be catalyzed by PA phosphatase. Further experiments indicated the presence of phospholipase D, acid phosphatase, and two different neutral lipases in extracts of cotyledons (data not shown).

Incorporation of Radioactive Precursors into Phospholipids. To find the extent to which phospholipids are synthesized in the cotyledons and to determine changes in phospholipid synthesis during seedling growth, slices of cotyledons were incubated with radioactive precursors of phospholipids. The phospholipids were extracted at the end of the labeling period, separated by TLC, and the incorporation into the individual phospholipids (PC, PE, and PI) determined. No effort was made to quantitate the incorporation into PA and other minor phospholipid species. The results of these incorporation studies are summarized in Table I. Data for acetate and glycerol incorporation are expressed as per cent of total uptake to correct for changes in the amount of available labeled precursor with seedling age, whereas the data for [³²P]Pi incorporation are expressed as per cent of incorporation into organic phosphate.

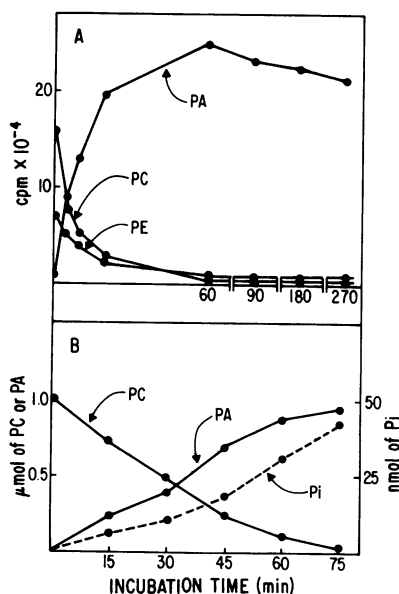


FIG. 2. Degradation of ³²P-labeled membranes (top) and ³²P-labeled PC in detergent micelles by enzymes present in a cotyledon homogenate. Top: membranes from 20 cotyledons were mixed with a cleared homogenate representing 1/2 cotyledon and incubated at 30°C. Bottom: 1 μmol of ³²P-labeled PC was incubated at 30°C with 1.5 mg of Nonidet P-40 and an aliquot of cleared cotyledon homogenate. Incubation for both experiments was at pH 5.3 (acetate buffer) and with 40 mM CaCl₂ to activate phospholipase D. Lipids were extracted with chloroform-methanol (2:1, v/v), separated by TLC and radioactivity in the individual phospholipids determined.

Incorporation of acetate was measured by incubating 10 sliced cotyledons obtained from seedlings of different ages for 60 min at 26°C in 2.0 ml of water containing 7 μCi of [¹⁴C]acetate. The uptake of precursor ranged from 40,000 to 130,000 cpm/cotyledon; incorporation into PC, PE, and PI together accounted for 3.5 to 5.0% of this total (Table I). Incorporation was relatively constant during the 5 days of seedling growth. It was not determined to what extent the acetate was incorporated into the fatty acid or the polar moiety of the phospholipids.

Incorporation of glycerol was measured using 3.6 μCi of [¹⁴C]glycerol per 10 sliced cotyledons. The uptake of [¹⁴C]glycerol by the tissue ranged from 18,000 to 45,000 cpm/cotyledon; incorporation into PC, PE, and PI together represented 15 to 18% of this total (Table I). Incorporation was again relatively constant throughout the period of growth of the seedling. Pi appeared to be most rapidly synthesized and incorporation into PI was highest 12 h after the start of imbibition.

Incorporation of [³²P]Pi was studied by incubating slices of 15 cotyledons for 60 min with 53 μCi of [³²P]Pi. The uptake of this precursor ranged from 400,000 cpm to 2,400,000 cpm/cotyledon and the incorporation into organic phosphates ranged from 275,000 cpm to 1,300,000 cpm/cotyledon. Only a small proportion of this radioactivity was accounted for by the phospholipids (6,000–12,000 cpm/cotyledon). Incorporation into PC and PE declined steadily from a high value 12 h after the start of imbibition, while incorporation into PI first increased, then decreased from a high value 24 h after the start of imbibition. To what extent these decreases in the incorporation of [³²P]Pi represent declining *de novo* synthesis or declining turnover of the polar moieties of the phospholipids cannot be determined from these data.

Enzymes for Phospholipid Biosynthesis. The rapid incorporation of phospholipid precursors as early as 12 h after the start of imbibition indicated that phospholipid-synthesizing enzymes must be active early in germination. This was confirmed by determining the activities of two such enzymes: phosphorylcholine-glyceride transferase and CDP-diglyceride-inositol transferase. The temporal changes in the activities of the two enzymes are shown in Table II. Both enzymes were present after 9 h of imbibition and increased about 2-fold in the next 12 to 36 h. This increase was followed by a decline in enzyme activity. We also measured the activity of these two enzymes in extracts of cotyledons obtained from beans which had been imbibed at 1°C (in an ice bucket) and found that the activities were nearly as high in cold-imbibed cotyledons as in room-temperature-imbibed cotyledons. These experiments indicate that these two phospholipid-synthesizing enzymes may preexist in the dry seeds and become activated during imbibition.

Table I. Incorporation of phospholipid precursors into major phospholipids during a 1-h incubation of sliced cotyledons in labelled precursor. Phospholipids were separated by thin layer chromatography. Incorporation of glycerol and acetate (isopropanol extraction) is expressed as % total uptake of label; incorporation of phosphate (chloroform:methanol extraction) is expressed as % incorporation into organic phosphates extracted as described by Saha and Good (20).

Precursor	Days of Growth	Incorporation into phospholipid		
		phosphatidylcholine	phosphatidylethanolamine	phosphatidylinositol
Acetate	1/2	2.12	1.29	0.27
	1	2.18	1.38	0.29
	2	1.05	1.69	0.45
	3	1.49	2.78	0.66
	4	1.68	1.59	0.63
Glycerol	1/2	6.73	4.53	9.37
	1	6.04	5.07	5.60
	2	4.59	6.48	7.19
	3	5.30	5.41	4.24
	Phosphate	1/2	0.58	0.95
1		0.35	0.81	0.09
1 1/2		0.15	0.49	0.08
2		0.08	0.30	0.05
3		0.03	0.24	0.05
4	0.04	0.30	0.09	

Turnover of Phospholipids in Cotyledons. The turnover of newly synthesized phospholipids was measured by labeling the cotyledons of intact plants with [^{14}C]glycerol for 2.5 h. The cotyledons of groups of 15 seedlings were immersed in 2 ml of water containing 2.3 μCi of [^{14}C]glycerol without detaching the cotyledons from the plants. Incorporation of [^{14}C]glycerol into the phospholipids of the cotyledons was assayed at the end of the labeling period and subsequently at 24-h intervals. The seedlings used in these experiments were 0.5, 2, or 4 days old. The total amount of radioactivity in the tissue at the end of the labeling period ranged from 15,000 cpm to 20,000 cpm/cotyledon. This level declined to 4,000 cpm to 6,000 cpm/cotyledon at the end of a 5-day period of seedling growth subsequent to the labeling with [^{14}C]glycerol. The combined incorporation into PC, PE, and PI ranged from 5,000 cpm to 7,000 cpm/cotyledon at the end of the labeling period and the decline of this radioactivity during subsequent seedling growth is shown in Figure 3. The data show that newly synthesized phospholipids were degraded with a half-life of 2 to 2.5 days whether they were synthesized when the seedlings were 0.5, 2, or 4 days old. Analysis of the individual phospholipids showed that PE and PC turned over with the same half-life (2–2.5 days). PI made 12 h after the start of imbibition was quite stable, while PI made later on also turned over in the same way as PE and PC (data for individual phospholipids not shown).

Newly Synthesized Phospholipids Associated with Subcellular Organelles. Sucrose gradients were used to find out in which organelles the newly synthesized phospholipids accumulated. Cotyledons were labeled with [^{14}C]glycerol and the homogenate layered on a 12 to 50% (w/w) sucrose gradient. After centrifugation to equilibrium most of the radioactivity was found in a broad peak with a mean density of 1.12 g/cm 3 (Fig. 4). The ER marker enzyme NADH-Cyt *c* reductase had the same density, but there

Table II. Phosphorylcholine : glyceride transferase (A) and CDP-diglyceride : inositol transferase (B) in the particulate fraction of cotyledons. Enzyme activity was measured in the particulate fraction recovered at the interphase of a 20%/35% discontinuous sucrose gradient.

Time from start of imbibition	temperature	Enzyme activity : pmoles/cotyledon	
		A	B
9	20 C	0.77	3.62
24	20 C	0.87	5.80
36	20 C	1.46	1.60
48	20 C	0.52	0.05
72	20 C	0.30	0.12
24	1 C	1.20	3.84

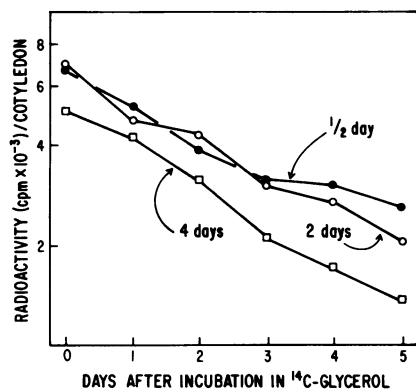


Fig. 3. Decay of ^{14}C label from [^{14}C]glycerol incorporated into phospholipids of cotyledons attached to intact seedlings. Seedlings were labeled for 2 h with radioactive precursor by inverting the seedlings so the cotyledons could be immersed in liquid. Seedlings were rinsed thoroughly and replanted in Vermiculite. Cotyledons were harvested at the times indicated and extracted with chloroform-methanol (2:1, v/v). Phospholipids were fractionated and incorporation into individual phospholipids was determined. Data shown here represent radioactivity in total chloroform-methanol extract, most of which could be accounted for by the three major phospholipids (PC, PE, and PI).

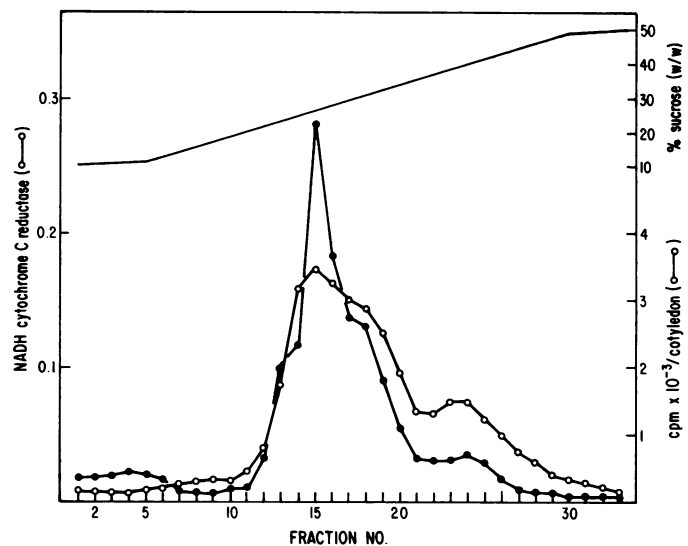


Fig. 4. Distribution of NADH Cyt *c* with reductase activity ($\Delta A_{550}/\text{min} \cdot \text{cotyledon}$) and [^{14}C]glycerol incorporation ($\text{cpm} \times 10^{-3}/\text{cotyledon}$) on a sucrose density gradient. Cotyledon slices were pulsed with [^{14}C]glycerol, homogenized and layered on a continuous sucrose gradient, 12 to 50% (w/w). After centrifugation to equilibrium fractions were assayed for enzyme activity and radioactivity.

was not exact coincidence between enzyme activity and radioactivity in glycerol. The radioactivity peak was broader than the enzyme peak. A minor peak of radioactivity was present at a density of 1.18 g/cm 3 . The low level of NADH-Cyt *c* reductase in this region of the gradient represents mitochondrial activity which is inhibited by antimycin A (data not shown). Protein body ghosts—protein bodies which have lost their protein—also band in this region of the gradient (Gilkes and Chrispeels, unpublished).

DISCUSSION

The data presented here show that germination and seedling growth in mung beans are accompanied by a rapid decline of the phospholipids in the cotyledons. The three major phospholipids, PC, PE, and PI, decline in parallel, starting 24 h after the beginning of imbibition. By the 4th day of growth the cotyledons have lost more than half their phospholipids. Although we cannot rigorously exclude the possibility that the phospholipids are being transported from the cotyledons to the axis, it seems reasonable to assume that the phospholipids are being catabolized in the cotyledons. Are the phospholipids part of the food reserves of the seed and do they constitute a source of phosphate for the growing axis, or is phospholipid catabolism an expression of cotyledon senescence? McKersie *et al.* (17) observed a similar decline in membrane-associated phospholipids in the cotyledons of *P. vulgaris* seedlings and postulated that this decline is an expression of organ and membrane senescence. In mung beans the timing of phospholipid catabolism is quite different from that of protein or RNA breakdown. The breakdown of stored protein requires the synthesis of vicilin peptidohydrolase. The synthesis of this protease normally begins on the 3rd day of growth and senescence of the first cells which have lost their protein reserves occurs on the 4th day of growth (2). Similarly, the decline of RNA in the cotyledons begins on the 4th day of growth (Chappell and Chrispeels, unpublished). The decline of phospholipids, on the other hand, begins 12 to 24 h after the start of imbibition. This indicates that the phospholipids may be stored reserves or may be part of a membrane system which was functional during seed maturation, but is not needed during seedling growth. We suggest that the breakdown of phospholipid is not an expression of the general senescence of the cotyledons.

Cotyledon homogenates contain enzymes capable of degrading membrane-associated phospholipids or phospholipids in micelles. When hydrolysis occurs in the presence of Ca^{2+} there is a rapid accumulation of PA and a slow release of water-soluble [^{32}P]phosphate. The accumulation of PA under the *in vitro* conditions may be due to the fact that phospholipase D was maximally activated by the addition of 40 mM CaCl_2 . Such a high concentration of CaCl_2 may not exist in the compartment where this hydrolysis occurs *in vivo*. Cotyledons obtained from beans imbibed for 24 h contained phospholipase D, acid phosphatase and two neutral lipases. The presence of these enzymes indicates that phospholipid catabolism may be able to start as soon as the beans are imbibed.

Cytological aspects of the interaction between the phospholipases and the phospholipids are presently being investigated and have been reported in preliminary form (11, 12). Most (70%) of the phospholipids are associated with the ER and/or other membranes which have a density of 1.12 g/cm³ in sucrose gradients, while phospholipase D and phosphatase are sequestered in the protein bodies. How the phospholipids in the ER come in contact with the hydrolases in the protein bodies remains to be determined.

The data reported here also show that the cells of the cotyledons of mung beans contain active phospholipid-synthesizing enzymes and are capable of rapid incorporation of radioactive precursors into phospholipids, soon after the start of imbibition. Phospholipid synthesis is one of the functions of the ER, and the ER can apparently resume or assume this function almost as soon as the seeds are hydrated. Cotyledons which have been imbibed for 12 h are rapidly synthesizing phospholipids.

Significant incorporation of acetate and glycerol into phospholipids occurs at all stages of seedling growth. Since the change in precursor pool size in the cotyledons was not measured one cannot draw conclusions about relative rates of synthesis; nevertheless there are no dramatic changes in the amounts of labeled acetate or glycerol incorporated, expressed as per cent of uptake, during seedling growth.

The incorporation of [^{32}P]Pi into the two major phospholipids (PC and PE) steadily declined when the data are expressed as a per cent of cpm in the organic phosphate fraction. This method of expressing the data corrects for changes in the inorganic phosphate pool and for changes in the entry of [^{32}P]Pi into organic phosphates (15). It is not clear, however, to what extent labeling with [^{32}P]Pi measures phospholipid biosynthesis or turnover of the polar head groups. That labeling by [^{32}P]Pi may reflect the turnover of polar head groups rather than the synthesis of phospholipids is indicated by results from several laboratories on the effect of GA_3 on phospholipid synthesis in barley aleurone cells. GA_3 enhances the incorporation of [^{32}P]Pi into membrane-bound phospholipids (15) without increasing the incorporation of glycerol (9), the synthesis of phospholipids (22), or the amount of ER in the tissue (7).

That mung bean cotyledons can synthesize phospholipids quite rapidly soon after imbibition is confirmed by the observation that they contain the phospholipid-synthesizing enzymes phosphorylcholine-glyceride transferase and CDP-diglyceride-inositol transferase. These enzymes are at near maximal activities 9 h after the start of imbibition and the activities found in beans imbibed at 1 C nearly equal those found in beans imbibed at 20 C. This observation suggests that the enzymes exist in the dry seeds. In this respect mung bean cotyledons appear to differ from some other storage tissues. In cucumber cotyledons (16) the enzymes for phospholipid biosynthesis are at very low levels 24 h after the start of seed imbibition and increase gradually during growth. The data for castor bean endosperm are somewhat contradictory. Bowden and Lord (3) reported low levels of enzyme activity and a gradual increase during seedling growth. Vick and Beevers (23) reported

a high level of phosphorylcholine glyceride transferase and the highest level of *sn*-glycerol-3-phosphate acylase 24 h after the start of imbibition. In mung bean cotyledons, on the other hand, activity is high to begin with and declines during seedling growth.

The newly synthesized phospholipids are associated with cellular membranes and turnover with a half-life of 2 to 2.5 days. Half-life does not depend on the time of synthesis and is the same whether the phospholipids were synthesized early in germination (12 h after imbibition) or later in seedling growth. Similar half-lives have been found by others for the phospholipids of plant cells, whether actively dividing (18) or not (8). We therefore suggest that these newly synthesized phospholipids may be participating in the normal biochemical and physiological processes of the cotyledons. The newly synthesized phospholipids are associated with organelles which have a density of 1.12 g/cm³ and 1.18 g/cm³ on sucrose gradients. These densities coincide with that of the ER (1.12 g/cm³) and of the mitochondria and protein body ghosts (1.18 g/cm³). The lack of exact coincidence between the radioactivity in glycerol and NADH-Cyt *c* activity indicates that membranes other than elements of the ER may be synthesized. The role of these membranes in the processes of reserve mobilization is presently under investigation.

LITERATURE CITED

- BARTLETT GR 1959 Phosphorus assay in column chromatography. *J. Biol Chem* 234: 466-468
- BAUMGARTNER B, KT TOKUYASU, MJ CHRISPEELS 1978 Localization of vicilin peptidohydrolase in the cotyledons of mung bean seedlings by immunofluorescence microscopy. *J Cell Biol* 79: 10-19
- BOWDEN L, JM LORD 1975 Development of phospholipid synthesizing enzymes in castor bean endosperm. *FEBS Lett* 49: 369-371
- CHRISPEELS MJ 1977 The role of the endoplasmic reticulum in the biosynthesis and transport of macromolecules in plant cells. In BR Brinkley, KR Porter, eds, *International Cell Biology 1976-1977*. Rockefeller University Press, pp 284-292
- CHRISPEELS MJ, B BAUMGARTNER, N HARRIS 1976 The regulation of reserve protein metabolism in the cotyledons of germinating legume seeds. *Proc Nat Acad Sci USA* 73: 3168-3172
- CHRISPEELS MJ, D BOULTER 1975 Control of storage protein metabolism in the cotyledons of germinating mung beans: role of endopeptidase. *Plant Physiol* 55: 1031-1037
- COLBORNE AJ, G MORRIS, DL LAIDMAN 1976 The formation of endoplasmic reticulum in the aleurone cells of germinating wheat: an ultrastructural study. *J Exp Bot* 27: 759-767
- CUMING AC, DJ OSBORNE 1978 Membrane turnover in imbibed dormant embryos of the wild oat (*Avena fatua* L.). II. Phospholipid turnover and membrane replacement. *Planta* 139: 219-226
- FIRN RD, H KENDE 1974 Some effects of applied gibberellic acid on the synthesis and degradation of lipids in isolated barley aleurone layers. *Plant Physiol* 54: 911-915
- FOLCH J, M LEES, GH SLOANE STANLEY 1957 A simple method for the isolation and purification of total lipids from animal tissue. *J Biol Chem* 226: 497-509
- GILKES N, N HARRIS, MJ CHRISPEELS 1978 Transformation of the endoplasmic reticulum in the cotyledons of mung bean seedlings. *Plant Physiol* 61: S-15
- HERMAN E, MJ CHRISPEELS 1978 Catabolism of phospholipids and compartmentation of phospholipases in cotyledons. *Plant Physiol* 61: S-15
- JOHNSON KD, H KENDE 1971 Hormonal control of lecithin synthesis in barley aleurone cells: regulation of the CDP-choline pathway by gibberellin. *Proc Nat Acad Sci USA* 68: 2674-2677
- KERN R, MJ CHRISPEELS 1978 Influence of the axis on the enzymes of protein and amide metabolism in the cotyledons of mung bean seedlings. *Plant Physiol* 62: 815-819
- KOEHLER DE, JE VARNER 1973 Hormonal control of orthophosphate incorporation into phospholipids of barley aleurone layers *Plant Physiol* 52: 208-214
- MACHER BA, CP BROWN, TT MCMANUS, JB MUDD 1975 Studies on phospholipid-synthesizing enzyme activities during the growth of etiolated cucumber cotyledons. *Plant Physiol* 55: 130-136
- MCKERSIE BD, JR LEPOCK, J KRUVU, JE THOMPSON 1978 The effects of cotyledon senescence on the composition and physical properties of membrane lipid. *Biochim Biophys Acta* 508: 197-212
- MOORE TS 1977 Phospholipid turnover in soybean tissue cultures. *Plant Physiol* 60: 754-758
- MOORE TS, JM LORD, T KAGAWA, H BEEVERS 1973 Enzymes of phospholipid metabolism in the endoplasmic reticulum of castor bean endosperm. *Plant Physiol* 52: 50-53
- SAHA S, NE GOOD 1970 Products of the photophosphorylation reaction. *J. Biol Chem* 245: 5017-5021
- SUMIDA S, JB MUDD 1970 The structure and biosynthesis of phosphatidyl inositol in cauliflower inflorescence. *Plant Physiol* 45: 712-718
- VARTY K, DL LAIDMAN 1976 The pattern and control of phospholipid metabolism in wheat aleurone tissue. *J. Exp Bot* 27: 748-458
- VICK B, H BEEVERS 1977 Phosphatidic acid synthesis in castor bean endosperm. *Plant Physiol* 59: 459-463