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Pyrimidine Metabolism in Cotyledons of Germinating Alaska Peas[1]

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ABSTRACT

Cotyledons from *Pisum sativum* L. cv. Alaska seeds were excised 12, 36, 108, 132, and 156 hours after imbibition in aerated distilled water. They were then incubated under aseptic conditions for 6 hours in solutions containing either uridine-2-^{14}C or orotic acid-6-^{14}C. Uridine was more extensively degraded to $^{14}CO_2$ at all germination stages than was orotate, and these rates remained essentially constant at each stage. Incorporation of each compound into RNA increased about 2-fold from the 12th to the 156th hour, although the total RNA present decreased slightly over this interval. Paper chromatography of soluble labeled metabolites produced from orotate showed that the capacity to metabolize this pyrimidine increased markedly as germination progressed. Radioactivity in uridine-5'-P, uridine diphosphate-hexoses, and uridine diphosphate increased most, while smaller or less consistent increases in uridine, uracil, uridine triphosphate, and an unidentified UDPX compound were also observed. The data suggest that orotate metabolism was initially limited by orotidine-5'-phosphate pyrophosphorylase or by 5-phosphoribosyl-1-pyrophosphate. Incorporation of uridine into RNA appeared to be limited at the earliest germination periods by conversion of uridine-5'-P to uridine diphosphate. Thus, during the 1st week of germination the orotic acid pathway and a salvage pathway converting uridine into RNA become activated.

Several studies concerning RNA or nucleotide metabolism in germinating pea seeds have been reported (2–6, 8, 9, 11, 12, 19). Barker and Douglas (2) reported that the RNA content of *Pisum sativum* cotyledons increased about 3-fold from the 1st to the 4th day and then decreased up to the 11th day. Later, however, Barker and Hollinshead (3) measured continuous decreases in RNA content of *Pisum arvense* cotyledons up to the 15th day. Beevers and Guernsey (5) studied RNA and other changes in cotyledons and embryos of germinating *P. sativum* seeds and observed little decline in cotyledonary RNA until about the 6th day. Their data are consistent with those of Bain and Mercer (1), who found little loss of storage products in the cotyledons until after about 1 week. It appears from ultrastructural, physiological, and biochemical changes measured by Bain and Mercer (1) that the 1st week represents a period in which pea cotyledons form active mitochondria and extensive endoplasmic and dictyosome membrane systems. These membranes might be needed for synthesis of enzymes to metabolize and transport food reserves to the embryos.

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Only limited information exists as to whether RNA synthesis occurs in pea cotyledons during germination. Barker and Hollinshead (3) found almost no incorporation of phosphate into *P. arvense* RNA up to the 15th day, although Beevers and Splittstoesser (6) reported that adenine was incorporated into RNA during the first 11 days. Beevers and Splittstoesser also found that adenine was converted into guanine, but no other soluble purine metabolites were found. Silver and Gilmore (19) infiltrated whole seedlings (6 day old) with various purine bases, nucleosides, or nucleotides. They found these compounds to be extensively metabolized, but only by hydrolytic, deamination, or oxidative reactions. No conversion of bases or nucleosides to nucleotides was detectable by their methods with unlabeled compounds. The data collectively suggest that little synthesis of nucleotides or RNA occurs in cotyledons of germinating peas. Our experiments were designed to determine whether the orotic acid pathway of pyrimidine nucleotide synthesis is functional during this time. We also wished to know whether uridine, possibly arising from reserve RNA by ribonuclease and nucleotidase action, could be reutilized for RNA synthesis or whether it is only degraded or transported to the embryos.

MATERIALS AND METHODS

Seeds of *Pisum sativum* L. cv. Alaska were soaked in 2 liters of distilled water through which filtered air agitating the seeds was continuously forced. The water (18–20 C) was changed every 12 hr and, except for the brief periods needed for these changes or to remove seeds for experiments, seeds were kept in darkness. Two similar experiments were performed. Twelve, 36, 108 (experiment 1), 132 (experiment 2), and 156 hr after the start of imbibition, samples were selected for metabolism studies and for fresh and dry (80–90 C for 24 hr) weight determinations.

Metabolism Studies. Seed coats were removed (if still present) and the two cotyledons were separated. Weights of the embryos were determined and embryos were then discarded. Cotyledons were surface sterilized by agitating them in 1% NaOCl for 2 min, followed by three successive rinses in sterile distilled water. Using sterile forceps, 10 cotyledons were placed flat (adaxial) side down in each of four autoclaved Skrip 2-ounce ink bottles with wells. To each well, 1.0 ml of 5% KOH was added to collect $^{14}CO_2$. The main compartment of each bottle contained 1.0 ml of autoclaved 0.02 M potassium phosphate buffer (pH 5.8) and 0.125 ml (1.5 μc) of either uridine-2-^{14}C (40 mc/mmole) or orotic acid-6-^{14}C (18 mc/mmole). Each labeled compound was obtained from the International Chemical and Nuclear Corp. Bottles were capped with autoclaved metal lids containing rubber gaskets to prevent loss of respiratory $^{14}CO_2$. Bottles were then placed on an oscillating water bath apparatus and incubated at 28 C for 6 hr under normal laboratory light.

Analysis of $^{14}CO_2$ was performed as described previously (15). The 10 cotyledons from each bottle were divided into two subgroups of five cotyledons each, these were rinsed extensively in water to remove unabsorbed orotic acid and uridine and were immediately frozen by immersion in an ethanol-Dry Ice bath for 5 min. Extraction of the metabolic products generally fol-

lowed the procedures described by Cole and Ross (10), using methods to minimize phosphatase action. Cotyledons were homogenized in 6 ml of 90% HCOOH in a mortar surrounded by an ethanol-Dry Ice bath. The homogenate was poured off, the mortar and pestle were rinsed with 10 ml of cold ethanol, and the HCOOH and ethanol solutions were combined. Each resulting homogenate was continuously stirred at 2 C for 10 min to further extract soluble pyrimidine metabolites and denature phosphatases. Homogenates were then centrifuged at 27,000*g* for 15 min at about 6 C to separate soluble nucleotides from insoluble nucleic acids.

Each supernatant solution was made to constant volume with 80% (v/v) ethanol and ^{14}C present was analyzed with a Nuclear-Chicago Corporation Model D-47 thin window gas flow geiger tube. Solutions were evaporated under vacuum at about 10 C to remove most of the ethanol and were then extracted with an equal volume of chloroform to remove material otherwise interfering with chromatographic procedures. The chloroform removed less than 1% of the ^{14}C present. The HCOOH-containing solutions were lyophilized and the residues were kept frozen prior to chromatography.

Each residue resulting from centrifugation of the tissue homogenates containing HCOOH-ethanol was extracted with 5 ml of hot ethanol-ether (2:1) to remove lipids. The resulting white powders were twice stirred with cold 0.2 N $HClO_4$ to remove soluble nucleotides not previously extracted. This step proved essential to prevent extensive contamination of RNA with the labeled orotic acid and uridine precursors and their metabolites. RNA was hydrolyzed in 0.3 M KOH and analyzed spectrophotometrically at 258 nm. Radioactivity in uridylic and cytidylic acids of the RNA was analyzed after removal of DNA and paper chromatography of the neutralized KOH hydrolysate (16).

Chromatographic Methods. Compounds in the lyophilized HCOOH-ethanol extracts were dissolved in water and aliquots were chromatographed on Whatman No. 3 MM papers using IBA (isobutyric acid–NH_4OH–H_2O, 57/4/39 v/v) followed by MAA (methanol–1 M ammonium acetate, 7/3 v/v) in the second direction. Separations obtainable with these solvents are shown in earlier publications (10, 18). Autoradiograms were prepared using Kodak Blue Brand x-ray film with exposure periods of 6 weeks. Spots thus detected were cut out of the papers and ^{14}C in each analyzed with a Nuclear-Chicago Mark I liquid scintillation counter.

Identities of most of the compounds could be tentatively made from their relative radioactivities and chromatographic positions compared to previous results of leaf and root studies (10, 18). Further confirmations were made by extracting the scintillation fluid residues from the paper spots with benzene, dissolving the labeled metabolites in water, and cochromatographing in the same and other solvents with known compounds. Identities of all except UDP-glucose, UDPX, and a compound or compounds migrating with UDP-glucuronic and UDP-galacturonic acids (slightly less rapidly than UTP in IBA and slightly more rapidly in MAA) were thus confirmed. UDPX is suspected to be a sugar derivative of UDP, since it migrated close to UDP-glucose in each solvent and was partially degraded to UMP during elution and rechromatography. The compound identified herein and in earlier papers (10, 17, 18) as UDP-glucose chromatographs with UDP-glucose, UDP-galactose, and UDP-xylose in these solvents. In no case have we determined what fraction of the total ^{14}C is in each of these closely related compounds.

RESULTS

The dry weight of each cotyledon decreased from about 85 mg to 64 mg (experiment 1) or 62 mg (experiment 2) over the 156-hr period. The embryonic dry weights were 2.0 mg at 12-hr imbibition, 2.4 mg at 36 hr, and had increased after 156 hr to 40 mg (experiment 1) or 45 mg (experiment 2). During this time the RNA per cotyledon decreased only slightly, from about 0.21 mg to 0.18 mg.

Minimal estimates of the orotic acid and uridine absorbed during 6-hr uptake periods during the various development stages are indicated in Figure 1. The values include the ^{14}C recovered in the CO_2, HCOOH-ethanol, ethanol-ether, $HClO_4$, and RNA fractions, but not the undetermined ^{14}C in DNA and KOH-insoluble material. The rate of orotate absorption increased during the first 36 hr of imbibition and then decreased after about 108 hr. Values for uridine absorption showed no consistent trend. About 40 to 54% of the uridine and 24 to 40%

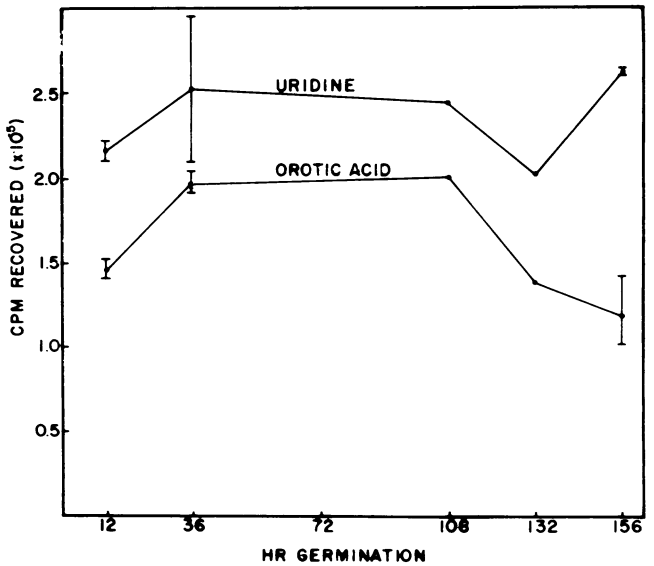

FIG. 1. Absorption of uridine-2-^{14}C and orotate-6-^{14}C by five pea cotyledons at various times after germination began. Absorption occurred during 6-hr periods at each time specified. Values are means from two experiments (individually indicated by vertical bars), except for the 108- and 132-hr periods.

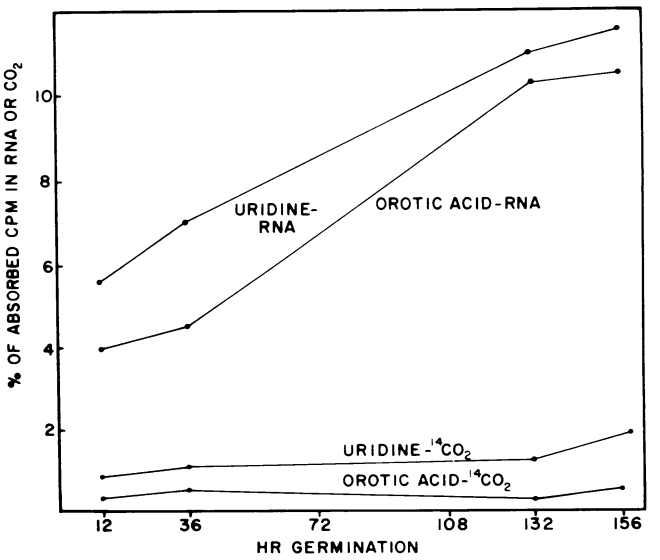

FIG. 2. Incorporation of orotate and uridine into RNA and their degradation to $^{14}CO_2$ at various germination stages. Values are means from both experiments except at 132 hr, and were calculated as percentages of ^{14}C absorbed given in Fig. 1.

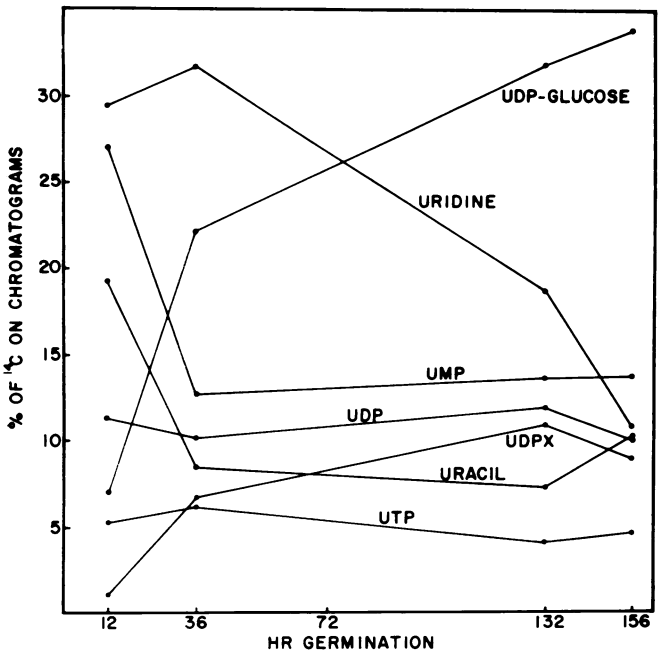

FIG. 3. Distribution of ^{14}C in uridine and HCOOH-ethanol soluble metabolites formed from uridine at different germination stages. Total detectable radioactivity upon the chromatograms (from which percentages in individual compounds were calculated) varied from about 10,000 to 20,000 cpm.

of the orotate provided was absorbed, depending on the growth stage.

The extent of catabolism to $^{14}CO_2$ and conversion into RNA for each compound at the various sampling times is shown in Figure 2. Because of differences in absorption rates, the values are expressed as percentages of total ^{14}C absorbed. On this basis uridine was catabolized to $^{14}CO_2$ considerably faster than was orotic acid, and no large changes in rates were found over the germination period studied. The two compounds were labeled in different positions, so these values do not necessarily indicate different rates of breakdown of the entire pyrimidine ring. We previously obtained evidence that orotate is catabolized in leaf tissues via uridine and uracil only after its conversion to UMP (15, 16).

Both RNA precursors were incorporated into cotyledonary RNA, even though no net RNA synthesis was occurring (Fig. 2). Thus, most of the enzymes of the orotate pathway are present, and a salvage pathway utilizing uridine is also operative even during the initial germination stages. However, incorporation increased considerably with development, and RNA labeling from uridine was always greater than from orotic acid. To learn whether such incorporation might simply represent the labeling of the terminal CCA group of transfer RNAs, all RNA samples from experiment 2 were hydrolyzed, chromatographed, and ratios of incorporation into cytidylate (C) and uridylate (U) residues were calculated. The C/U ^{14}C ratios were usually between 0.2 and 0.3 and were similar at all stages, showing that true RNA synthesis took place. These results also indicated the presence of small quantities of contaminating ^{14}C migrating between the C and U spots. Thus the RNA-^{14}C values in Figure 2 are slightly overestimated.

Results from chromatographic separations of metabolites in the HCOOH-ethanol extracts from the second uridine experiment are indicated in Figure 3. Very similar results were obtained in the first experiment. These values do not necessarily represent true estimates of the tissue contents, since some destruction probably occurred during extraction, and because nucleotides were undoubtedly present in the $HClO_4$ extracts. The latter were not chromatographed because of $KClO_4$ interference. At both the 12- and 36-hr periods uridine was the principal labeled compound detected. The percentage of ^{14}C in this compound decreased rapidly at subsequent times, consistent with its increased degradation to CO_2 and incorporation into RNA as development progressed.

The ^{14}C in uracil did not increase with development, but initially showed a rapid decrease. The same was true for UMP. This decrease in UMP labeling was apparently largely accounted for by an increased formation of UDP-glucose and other UDP-sugars which chromatograph with it (see "Materials and Methods") and of UDPX. The identity of UDPX is unknown. Brown (8) found amounts of a UDP-sugar later identified as UDP-fructose (9) to increase in cotyledons of germinating peas. This compound had the same chromatographic properties in various solvents (including IBA and ethanol-ammonium acetate) as did UDP-glucose. It is, therefore, likely that the compound we have identified as UDP-glucose also contains UDP-fructose. UDPX might contain UDP-rhamnose, since this compound was found in fresh pea seeds by Hampe and Gonzalez (11), and it had a chromatographic mobility in ethanol-ammonium acetate relative to UDPG of 1.1, which is the same as we observed for UDPX in methanol-ammonium acetate.

The most striking result of these experiments was the increased conversion of uridine to UDP-glucose and UDPX as development proceeded. In spite of the rise in conversion of uridine into RNA (Fig. 2), UDP- and UTP-^{14}C values remained rather constant at each stage. Perhaps this reflects the net result of an increased formation of these nucleotides from uridine and UMP, along with an increased conversion to RNA and UDP-sugars.

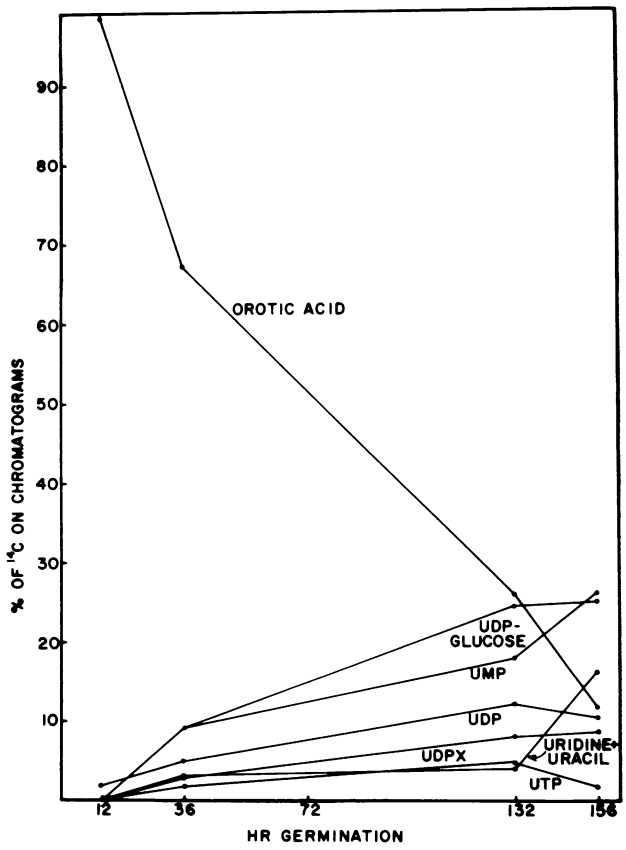

FIG. 4. Distribution of ^{14}C in orotate and HCOOH-ethanol soluble metabolites formed from orotate at various germination stages. Total radioactivity recovered in individual compounds upon a chromatogram averaged about 9,000 cpm.

Cytidine nucleotides were never detected as products of uridine metabolism, with the exception of the occasional occurrence of a compound with the mobility of CMP on the chromatograms. Nevertheless, the detection of labeled cytidylate in the RNA hydrolysates indicates that CTP, at least, was formed. Brown (8) also was unable to find significant amounts of cytidine compounds in dry or germinating pea seeds.

Figure 4 shows the changing patterns of HCOOH-ethanol soluble products from the second orotic acid incorporation experiment as germination progressed. At 12 hr nearly all ^{14}C extracted remained in unmetabolized orotate. Similar results were obtained in the first experiment. During later periods the cotyledons developed increased capacity to convert orotate to nucleotides. Thus UMP, UDP-glucose, UDP, UTP, and UDPX all were labeled more extensively by seeds germinated 132 hr than only 12 hr. Both uracil and uridine were found on most of the chromatograms from later germination periods. The ^{14}C present in these two compounds varied considerably among chromatograms even of similar treatments, suggesting that they may have arisen in part from degradative reactions during extraction or concentration of the extracts. Radioactivity in a spot with the same mobility as UDP-hexuronic acids was found in traces on chromatograms from later germination stages (as was also true in the uridine experiments), and again CMP was the only cytidine nucleotide containing detectable ^{14}C (data not plotted).

The predominance of orotate on chromatograms from extracts made after the 12- and 36-hr imbibition periods is consistent with the relatively weak incorporation of orotate into RNA at these times (Fig. 2). Since some RNA labeling and degradation to CO_2 occurred then, nucleotides must have been present in small amounts.

DISCUSSION

The results indicating a decrease in cotyledon dry weight and a small loss of RNA during the first 6 days of germination are very similar to those of Beevers and Guernsey (5). The total RNA content observed in the present work (0.26% of dry wt) was also very close to that calculated from their data (0.28% of dry wt), even though different varieties and extraction methods were used. Bain and Mercer (1) concluded from electron microscopic investigations and chemical analyses that little transport of nitrogenous or other materials occurred from Victory Freezer pea cotyledons during the first 6 days, although breakdown of starch and protein commenced with imbibition. They observed an extensive development of endoplasmic reticulum, dictyosome membranes, and active mitochondria during this period, with loss of food reserves and senescence occurring only later.

The incorporation of both orotate and uridine into RNA shows, contrary to previous interpretations from *P. arvense* (3, 4), that RNA synthesis does occur in pea cotyledons during germination. Beevers and Splittstoesser (6) also obtained results indicating that adenine-^{14}C is incorporated into RNA of cotyledons. We have found, however, that ethanol extraction techniques similar to those they used leave significant proportions of soluble nucleotides remaining in the RNA fraction. Contamination of RNA with labeled adenine and free nucleotides might account for the relatively high and variable ^{14}C values in RNA compared to the ethanol extracts in their studies.

Both orotate and uridine were converted into RNA in increasing amount as germination progressed. This was not due to differences in rates of absorption by the cotyledons, but might be explained by development of the capacity to convert both compounds to UMP and other nucleotides. For orotate the limiting reaction at early stages was likely its conversion to UMP. Formation of OMP[2] by OMP-pyrophosphorylase may have been the critical step, since the absence of detectable OMP indicates it was rapidly converted to UMP. Whether OMP-pyrophosphorylase might be activated or synthesized during germination or whether PRPP is initially limiting is under investigation. Brown and Wray (7) showed that activity of the pentose phosphate respiratory pathway increased greatly over the first 6 days of germination in Meteor pea cotyledons. This pathway produces the ribose-5-P presumably necessary for PRPP-synthesis, so such an increase might account for the increased orotate anabolism we observed. It would be surprising to find that OMP-pyrophosphorylase is the limiting enzyme of the entire orotate pathway here, since free orotate was not detected in dry or imbibed seeds (8). It is more likely that one or more enzymes functioning earlier in the pathway are at first inactive.

The low incorporation of uridine into RNA at 12 and 36 hours was not due to its poor conversion to UMP, since UMP contained almost as much ^{14}C as did uridine in the 12-hr seeds. The increased importance of UDP-sugars at later stages and decrease in the UMP curve between 12 and 36 hr suggests that conversion of UMP to UDP may initially have been rate limiting. If so, the UMP-kinase enzyme required might have been inactive, because the levels of ATP presumed essential were apparently high enough to allow UMP formation from uridine even at 12 hr. Brown (8) found that the ATP content of whole Meteor pea seeds increased 250% during the first 16 hr of germination, although others (7) found a steady decrease over the first 8 days in cotyledons of the same variety.

The possibility that conversion of uridine and orotate into RNA was at first limited by the activity of RNA polymerase was considered. A similar explanation has sometimes been offered to account for hormone-stimulated conversion of pyrimidines into RNA of excised plant tissues, yet analyses of labeled nucleotides have apparently not been reported in such experiments. Our results showed that pea cotyledons contained essentially constant amounts of labeled UTP at all times. Thus UTP levels had no apparent relation to the extent of RNA labeling and UTP did not accumulate as it might have if RNA polymerase were limiting.

The decrease of ^{14}C found in uridine and UMP during germination in uridine experiments is consistent with Brown's results (8). He reported that the amounts of uridine and UMP in pea seeds decreased about half during the first 40 hr of germination. However, he observed a slight decrease in the amounts of UDP-glucose and UDP-galactose (unseparated), while we found that amounts of ^{14}C in what also likely contains a mixture of these two UDP-sugars greatly increased during this time. An increased production of UDP-glucose is consistent with the known increases in reducing sugars (1, 7) and sucrose (13) occurring during germination of peas. UDP-glucose is presumably important in synthesis of sucrose transported to the growing embryo, while both UDP-glucose and UDP-galactose could be involved in formation of certain glycolipids presumably essential to development of membrane systems occurring in cotyledon cells (1). Formation of Golgi and endoplasmic reticulum membranes might be necessary for subsequent transport of substances from the cotyledons to the embryos.

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[2] Abbreviations: OMP: orotidine-5′-P, PRPP: 5-phosphoribosyl-1-pyro-P.

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