Phospholipids in the Uredospores of *Uromyces phaseoli*

**II. METABOLISM DURING GERMINATION**

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**ABSTRACT**

The levels and types of phospholipids changed in distinct phases during the germination of ³²P-labeled uredospores of *Uromyces phaseoli*. During the first 20 minutes of germination, the phospholipid content dropped to 40% of the pregermination level. Between 2 and 3 hours, phospholipid levels increased to approximately 80% of the pregermination levels, and after germination for 5 hours, catabolism had reduced the ³²P-lipids to about the same level observed prior to the first anabolic phase. A second anabolic phase was observed between 5 and 10 hours of germination. Phosphatidylethanolamine and phosphatidylethanolamine, the major phospholipids, did not undergo anabolism and catabolism at the same rates during germination. Only small quantities of the more polar phospholipids were released to the germination media.

Germinating uredospores were capable of utilizing L-methionine-methyl-¹⁴C, D, L-¹³C serine, ¹⁴C-histine, and ¹³C-ethanolamine for the synthesis of phospholipids. The active one-carbon units from methionine and serine appear to be involved in the methylation of phosphatidylethanolamine to form phosphatidylethanolamine. Preformed ethanolamine and choline may be incorporated into these two phospholipids also. Evidence for the synthesis of phosphatidylethanolamine, which is subsequently deoxygenylated to yield phosphatidylethanolamine, was obtained.

Detailed studies concerning the metabolism of individual phospholipids in germinating spores of obligately parasitic fungi have not been previously reported. Although the biosynthetic pathways for the major phosphatides in animal and bacterial systems have been extensively studied, relatively little is known concerning their biosynthesis in higher plants and fungi. Recently, Kates (7) reviewed the subject of plant phospholipids.

While the germination process for uredospores of fungi has been studied extensively, little is known concerning the actual synthesis of the germ tube and associated membranes. This work was initiated to elucidate the possible pathways for the synthesis of phospholipids and to investigate whether possible relationships existed between phospholipid metabolism and structural changes.

**MATERIALS AND METHODS**

**Uredospore Production.** The production of ³²P-labeled and unlabeled uredospores have been described (11, 18).

**Germination of Spores.** Immediately before germination the spores were washed with distilled water, air dried (18), and then weighed. Germination was initiated by dispersing the spores evenly on the surface of the medium (1.0 mg spores per 1.0 ml medium). Depending upon the quantity of spores required, either Petri dishes (9.4 cm diameter) or Pyrex trays (18.5 x 30 x 4 cm) were used as germination vessels. The medium was distilled water, except when the incorporation of specific substrates was being studied. In those cases the medium was a distilled water solution of the appropriate compound.

Germination was terminated by filtering the spores and medium through a glass wool plug. The glass wool plug and entrapped spores were then immersed in a chloroform-methanol (2:1 v/v) mixture. The germination period was measured from the time the spores were dispersed on the medium to the time the spores were immersed in the chloroform-methanol mixture.

**Phospholipid Extraction.** The methods used in disrupting the spores and extracting the phospholipids are described in the previous paper (11).

**Phospholipid Analysis of Spores.** As a measure of total phospholipids the radioactivity (³²P or ¹³C) of the chloroform-methanol extract was determined. For the determination of radioactivity of specific phospholipids, the chloroform-methanol extract, or an aliquot of the extract, was subjected to thin layer chromatography. The radioactive bands were located by autoradiography or by spraying a margin of the thin layer plate with one of the reagents given previously (11). The adsorbent from the desired area was scraped directly into a counting vial. After adding 1 ml of ethanol and agitating the sample for 10 min, 15 ml of the scintillation solution (dioxane-xylene solvent) was added. If a detecting reagent was used, only the unspayed region of the plate was used for analysis. For ³²P-lipids (located by autoradiography) the counting rates reported were only corrected for decay and the proportion of the extract applied to the plate. By this method more than 90% of the radioactivity (cpm) applied to the chromatogram could be accounted for by the combined radioactivity (corrected cpm) of the components. For ¹³C-lipids (located by spray reagents) the percentage of each component was calculated on the basis of cpm. The percentage obtained was multiplied by the total radioactivity of the extract (determined by counting an aliquot and applying a channels ratio quench correction) to obtain the...
radioactivity of each component. The areas of the chromatograms that did not contain compounds were also scraped and assayed, and the data were included in the percentage calculations.

Phospholipid Analysis of the Germination Medium. The quantity of phospholipids which were present in the germination medium was determined by lyophilizing the medium and extracting the dry residue with chloroform-methanol (2:1, v/v). Care was taken to insure that no ureidospores were present in the filtered medium prior to the lyophilization step. The radioactivity in the extract was considered as the radioactivity of the total phospholipids. Assay of specific phospholipids was performed by the same methods described in the previous section.

Thin Layer Chromatography. Thin layer chromatography was performed with Silica Gel G as the absorbent. The preparation of the plates has been described previously (10). The solvent systems used were system A, chloroform-methanol-water (66:30:4, v/v/v) (15), and system D, chloroform-methanol-7.4 N ammonium hydroxide (66:17:3, v/v/v) (19).

Chemicals. Reference compounds and solvents were from the same sources previously reported (11). Phosphoric acid-\(^{32}P\) (carrier free), 1-methionine-methyl-\(^{14}C\) (10.6 mc/m mole), l-serine-3-\(^{14}C\) (4.8 mc/m mole), choline-1, 2-\(^{14}C\) chloride (5 mc/m mole), and ethanolamine-1, 2-\(^{14}C\) hydrochloride (1.5 mc/m mole) were purchased from New England Nuclear, Waltham, Massachusetts.

RESULTS

Metabolism of Endogenous Phospholipids. Uredospores of Uromyces phaseoli, previously labeled with \(^{32}P\), were germinated on distilled water for various time periods, and the total intracellular phospholipid content was determined. The amount of phospholipid released to the germination medium was also analyzed.

The levels of phospholipids appear to change by distinct phases during the germination process (Fig. 1). The total \(^{32}P\) in the chloroform-methanol extract of the spores decreased by approximately 50% during the first 20 min germination period. In the same time period, a slight swelling of the spores was the only physical manifestation of the germination process, and protrusion of the germ tube was not evident until approximately 40 min after the germination was initiated. Between 2 and 3 hr the phospholipid levels increased to about 80% of the initial level. After germination for 5 hr, catabolism had reduced the \(^{32}P\)-lipids almost to the levels observed prior to the anabolic phase. Synthesis of phospholipids appeared to occur again between 5 and 10 hr, and in all studies the maximum level of \(^{32}P\) in the chloroform-methanol extract was observed at about 10 hr. Other experiments performed in this laboratory have indicated that an additional catabolic phase followed by an anabolic phase occurred between 10 and 102 hr of germination. Also shown in Figure 1 is the amount of phospholipid released to the medium as the germination progressed. Although the phospholipid levels of the medium appeared to change in an inverse manner with the phospholipid levels in the spores, the difference in the levels of the medium was not nearly great enough to account for the changes in phospholipid level of the germinating ureidospores. Therefore, the disappearance of chloroform-methanol extractable \(^{32}P\) during the first 20 min of germination represented metabolism of the phospholipids and not the release of phospholipids to the germination medium.

The successive anabolic and catabolic phases of phospholipid metabolism were not expected; however, the experiment was repeated several times and in all studies the same basic metabolic phases were observed. In one experiment, duplicate samples were germinated for each of the time periods. The duplicate samples agreed within \(\pm 10\%\), and illustrated that the early catabolic and anabolic phases of phospholipid metabolism were real and not variations in analyses.

The levels of individual phospholipids at different stages of germination were investigated by subjecting the chloroform-methanol extracts to thin layer chromatography in chloroform-methanol-water (66:30:4, v/v/v), (system A) and determining the radioactivity in the separated components. From the chromatographic mobilities of the components and the identification of the phospholipids in previous work (11), the identity of the components was determined. Figure 2 depicts the changes...
in the individual phospholipid levels during the germination process. Diphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine all decreased during the first 20 min germination period. More dramatic, however, was the decrease in the unidentified $^{32}$P-labeled material which remained at the origin in this chromatography system. Although the identity of these $^{32}$P-labeled components was not elucidated, at least one was believed to contain choline. The levels of the two phosphatidylinositides and of diphosphatidylglycerol did not show a definite change during the first 20 min period. In the first major anabolic phase (between 2 and 3 hr), all of the phospholipid levels increased; however, phosphatidylethanolamine and the $^{32}$P-labeled material at the origin of the chromatographic plate showed the most pronounced increases. On a percentage basis, the level of the phosphoinositides increased significantly. The decrease in total phospholipids in the second catabolic phase (between 3 and 5 hr) appears to be largely due to changes in phosphatidylethanolamine and unidentified $^{32}$P material which increased the most during the previous anabolic phase. The second major anabolic phase (between 5 and 10 hr) appears to be the result of synthesis of all phospholipid components, with phosphatidylethanolamine contributing the most to the increase in total phospholipid levels. Diphosphatidylglycerol, which is a minor component of the phospholipids, did not undergo distinct changes in level. While the level does appear to fluctuate with the total phospholipid level, the differences could not be considered to be significant when the precision of the assay method is considered. In Figures 1 and 2 there is an indication that phospholipid anabolism took place between 20 min and 1 hr and that a catabolic phase follows between 1 and 2 hr.

Analysis of the phospholipids in the filtered media indicated that the more polar phospholipids, phosphatidylethanolamine, the two inositol-containing phosphatides and the unidentified $^{32}$P-labeled material which remained at the chromatographic origin were preferentially released. Very little phosphatidylethanolamine was present in the germination medium regardless of the period of incubation. The phospholipids in the spores and those in the medium at the end of a 2 hr germination period are shown in Figure 3, which is a photograph of an autoradiogram obtained by chromatography of the chloroform-methanol extracts of the germinated spores and the lyophilized medium. It appears that those phospholipids that would be expected to have the greatest solubility in water were present in the highest concentrations in the germination medium. This does not rule out the possibility that other phospholipids were transported from the intact spore, but instead of dissolving in the medium, adhered to the spore wall or germ tube.

The results of the experiments described thus far indicate that large changes in phospholipid levels occur during the germination process and that these changes are primarily due to the anabolism and catabolism of phosphatidylcholine, phosphatidylethanolamine, and the $^{32}$P-labeled material that remains at the origin when the phospholipids are chromatographed. Therefore, in addition to changes in the phospholipid level, there also appear to be changes in the types of phospholipids present during different stages of the germination process.

**Incorporation of Methionine-methyl-$^{14}$C into Phospholipids.**

The anabolic phases observed at various stages of the germination process indicated that pathways for the biosynthesis of phospholipids, particularly phosphatidylcholine and phosphatidylethanolamine, existed in the germinating spore. For other biological systems, two pathways which would achieve a net synthesis of phosphatidylcholine are known: the successive methylation of phosphatidylethanolamine to yield phosphatidylethanolamine, and the condensation of CDP-choline with an $\alpha,\beta$-diglyceride (13). In the preceding report (11), it was shown that labeled phosphatidylcholine, phosphatidyl dimethylethanolamine, and phosphatidylmonomethylethanolamine were synthesized when spores were germinated on media containing methionine-methyl-$^{14}$C. In those experiments, methionine was readily taken up by spores since up to 30% of the radioactivity originally present in the medium could be extracted from germinated spores with chloroform-methanol after a 10 hr germination period.

![Fig. 3. An autoradiogram showing the thin layer chromatographic separation of the chloroform-methanol extractable, $^{32}$P-labeled components of the germination medium and germinated ureidospores of *Uromyces phaseoli*. The germination period was 2 hr and chloroform-methanol-water (66:30:4, v/v/v) was used as the chromatography solvent. Approximately equal quantities of $^{32}$P-radioactivity were applied to the thin layer chromatography plate. The origin and solvent front are indicated by OR. and SF., respectively. DPG: diphosphatidylglycerol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; PI: phosphatidylinositol; UN: an unidentified inositol-containing phospholipid.](image-url)
The incorporation of the $^{14}$C-labeled methyl group of methionine into individual phospholipids was determined at different stages of germination. The experiments were carried out by germinating 20 mg of uredospores for various periods of time. The methionine concentration was 10 μM, and each Petri dish contained 4.4 × 10$^6$ dpm of radioactivity. Chromatographic separation of the individual phospholipids was accomplished by system D. During the first 10 hr, a linear rate of incorporation of the labeled methyl group into phosphatidylcholine was observed. Knowing the specific radioactivity of the labeled methionine, it was calculated that about 33 nmol of the methionine methyl groups were incorporated into phosphatidylcholine during the 10 hr germination period. Therefore, approximately 17% of the methionine originally present in the medium was utilized in the synthesis of phosphatidylcholine. Phosphatidylmonomethylethanolamine and phosphatidylmethyllethanolamine appeared to reach a low, steady state level in the early stages of the germination. These studies indicate that the successive methylation of phosphatidylethanolamine to synthesize phosphatidylcholine does occur in germinating uredospores of *Uromyces phaseoli*. The methylation of phosphatidylethanolamine to form phosphatidylcholine occurred at a continuous rate over a 10-hr period, yet changes in phosphatidylethanolamine levels were observed when $^{32}$P-labeled uredospores were germinated. In considering several explanations, it seemed likely that in the catabolism of phosphatidylcholine, choline was not degraded and could be reincorporated. Therefore, a pathway for the synthesis of phosphatidylcholine from choline and α,β-diglyceride would be expected to exist.

**Incorporation of $^{14}$C-Choline and $^{14}$C-Ethanolamine into Phospholipids.** To determine if preformed choline and ethanolamine could be incorporated into phosphatidylcholine and phosphatidylethanolamine, respectively, uredospores were germinated on medium containing the appropriate labeled substrates. The germination period was 10 hr and 20 mg of spores were used per sample. Concentrations of the radioactive substrates were: choline-1,2-4$^14$C, 20.0 nmol/ml (2.32 × 10$^6$ dpm/ml) and ethanolamine-1,2-4$^14$C, 1.59 × 10$^6$ nmol/ml (5.3 × 10$^4$ dpm/ml). System A was used in the chromatographic separation of the phospholipids. The data obtained are shown in Table I. Neither choline nor ethanolamine was readily taken up by the germinating uredospores. Only 0.35% of the radioactivity in the $^{14}$C-choline-containing medium was present in the chloroform-methanol extract of the spores that germinated on that medium. However, approximately 80% of the $^{14}$C-choline in the spore was in the form of phosphatidylcholine. The remainder of the radioactivity did not move from the origin of the chromatogram and was believed to be choline or its derivative.

On the basis of radioactivity, approximately 0.52% of the ethanolamine in the medium was extracted with chloroform-methanol from the germinated spores, and about 74% of that radioactivity was associated with phosphatidylethanolamine. Apparently, $^{14}$C-labeled phosphatidylethanolamine was also synthesized by the methylation of phosphatidylethanolamine, or perhaps, ethanolamine.

It appears that choline and ethanolamine can be utilized in the biosynthesis of phospholipids. Presumably, the anabolic pathway which involves an α,β-diglyceride and the appropriate CDP derivative, CDP-choline or CDP-ethanolamine, does function in the germinating uredospores of *Uromyces phaseoli*.

**Incorporation of $^{14}$C-Serine into Phospholipids.** Besides the reaction of CDP-ethanolamine with an α,β-diglyceride, the synthesis of phosphatidylethanolamine may also occur, in some organisms, by the decarboxylation of phosphatidylserine. To determine if the latter pathway was operative in germinating uredospores, spores were germinated on media containing D,3-serine-3-2$^14$C. For each sample 20 mg of spores were germinated 10 hr. The concentration of $^{14}$C-serine was 43.9 nmoles/ml (4.68 × 10$^6$ dpm/ml). In one experiment 2 mm choline was included in the medium and in another 1 mm methionine was included. System A was used in the chromatographic separation of the lipids.

Serine was fairly readily incorporated into lipids, as 3.21% of the $^{14}$C-activity in the medium was extractable from the germinated spores with chloroform-methanol. The presence of choline in the medium appeared to stimulate the incorporation of serine slightly, whereas when 1 mm methionine was in the medium, the incorporation of $^{14}$C-serine into lipids by the germinating spores was only 0.064%. Table II shows the quantity of radioactivity incorporated into each of the components of the chloroform-methanol extract of the germinated spores. With the three types of media, the $^{14}$C of serine was incorporated into phosphatidylethanolamine, phosphatidylcholine, and a neutral lipid component which consisted of C8 sterols (unpublished data).

The $^{14}$C of serine could be incorporated into phosphatidylcholine by two pathways. The decarboxylation of phosphatidylserine would yield phosphatidylethanolamine and hence phosphatidylcholine, which would possess the second and third carbon atoms of serine. Methylolation of phosphatidylethanolamine with active one-carbon units from serine would also incorporate the $^{14}$C-labeled carbon atom of serine into phosphatidylcholine. It appears that both routes of incorporation were operative when the data of Tables I and II are considered. The ratio of the radioactivity in phosphatidylethanolamine and phosphatidylcholine was 4.9 to 1 when $^{14}$C-ethanolamine was used as a substrate. With $^{14}$C-serine as a substrate the same ratio was 1.6 to 1. The ratios were 2.7 to 1 and 1.3 to 1 when $^{14}$C-serine was the substrate in media also containing 2 mm choline and 1 mm methionine, respectively. If the only route of incorporation of serine-2-2$^14$C into phosphatidylethanolamine was via phosphatidylethanolamine, one would expect the ratios of the activity to be the same when either ethanolamine-2$^14$C or serine-

### Table I. Incorporation of Radioactivity into Components of the Chloroform-Methanol Extract of Uromyces phaseoli 14C-Choline and 14C-Ethanolamine

<table>
<thead>
<tr>
<th>Component</th>
<th>Radioactivity Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{14}$C-choline</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>0</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>12,800</td>
</tr>
<tr>
<td>Chromatographic origin</td>
<td>3,200</td>
</tr>
</tbody>
</table>

### Table II. Incorporation of Radioactivity into Components of the Chloroform-Methanol Extract of Uromyces phaseoli 14C-Serine

<table>
<thead>
<tr>
<th>Component</th>
<th>Radioactivity Incorporated</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$^{14}$C-serine</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>72,000</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>45,000</td>
</tr>
<tr>
<td>Steroids</td>
<td>183,000</td>
</tr>
</tbody>
</table>

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\(^{14}\text{C}\) was the substrate in the medium. Phosphatidylserine is apparently formed in the germinating uredospore, but functions only as an intermediate in the synthesis of phosphatidylethanolamine and phosphatidylcholine. Serine may also serve as a source of active one-carbon units for the methylation of phosphatidylethanolamine.

**DISCUSSION**

Phospholipids in the spore appear to be extensively degraded in the very early stages of germination and then resynthesized at later stages. The various phases indicate there are changes in the function of phospholipids during the germination period.

Whereas the biosynthetic pathways for phospholipids have been studied in mammalian and bacterial systems, our knowledge in plant tissue is incomplete. From this investigation it appears that germinating uredospores of *Uromyces phaseoli* are capable of catalyzing the successive methylation of phosphatidylethanolamine to form phosphatidylethanolamine by the utilization of the methyl group of methionine. This pathway has been recently demonstrated in *Saccharomyces cervisiae* (16, 19). On the basis of our incorporation studies, the condensation of choline, ethanolamine, and serine with diglyceride to form, respectively, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine does occur, although the intermediates involved have not been identified. In animal tissues, CDP-choline and CDP-ethanolamine react with a diglyceride to form the phosphatides (8, 9) while \(L\)-serine condenses with CDP-diglyceride to yield phosphatidylserine in bacterial systems (6). The decarboxylation of phosphatidylserine to give phosphatidylethanolamine appears to take place in germinating uredospores. Serine also furnishes active one-carbon units for the synthesis of phosphatidylethanolamine as well as the steroids that are formed during germination.

The pattern of phospholipid levels observed in this study is similar to that reported for germinating spores of *Aspergillus niger* (14). In *Aspergillus*, the rapid catabolism of phospholipids was concomitant with an increase in sugar phosphates and nucleotides, with some of the phosphorus being incorporated into RNA.

Jackson and Frear (5) determined total lipid phosphorus levels in germinating flax rust uredospores (*Melampsora lini*) at 2-hr intervals and reported a continuous increase of lipid phosphorus during the first 6 hr of germination. It is possible that an initial catabolism of phospholipids also occurs in this organism, but the first anabolic phase restores the level of phospholipids within the first 2-hr period instead of the 2.5- to 3-hr period observed in *Uromyces phaseoli*. However, the other fluctuations of lipid phosphorus levels were not observed.

The oxygen uptake studies reported by Maheshwari and Sussman (12) closely parallel the catabolism of phospholipids observed in this study with the periods of greatest oxygen consumption being concomitant with the periods of greatest phospholipid catabolism.

The changes in phospholipid levels may reflect a degradation and resynthesis of membranes. Ultrastructural studies (17) indicate that as early as 0.5 hr after germination has commenced, morphological changes are visible in *Puccinia graminis tritici*. The electron transparent patches of the cytoplasm almost occupy the cytoplasm after 1.5 hr of germination. On a time scale, the appearance of these transparent regions correlates well with the initial catabolism of phospholipids by *Uromyces phaseoli*. Williams and Ledingham (20) have reported that the diameter of the lipid bodies in ungerminated spores is 0.8 \(\mu\) and that the diameter of lipid bodies in the germ tube is about 0.1 \(\mu\). It has been shown in spores of *Mucor rouxii* that hyphae elongate by tip growth and that the most active synthesis of hyphae occurs at the apical segment (2, 3). Assuming a similarity in germ tube growth, the greatest need for energy and precursors of the cell wall would be at the apical portion of the elongating germ tube. In spores of rust fungi, the greatest utilization of lipids appears to occur during the time the germ tubes are rapidly elongating (4). Perhaps a function of phospholipids is the formation of membranes which surround the small lipid droplets for their transport through the germ tube.

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**LITERATURE CITED**