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# PHOSPHATIDYLINOSITOL MONOPHOSPHATE IN *LILIUM* POLLEN AND TURNOVER OF PHOSPHOLIPID DURING POLLEN TUBE EXTENSION

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**Key Word Index**—*Lilium longiflorum*; Liliaceae; *myo*-inositol; phosphatidylinositol; phosphatidylinositol monophosphate; phospholipid; pollen.

**Abstract**—A considerable incorporation of [$^{32}$P]orthophosphate into phospholipids was observed during germination of pollen of *Lilium longiflorum*, particularly during the period of most rapid pollen tube elongation. The major phospholipids were phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol and phosphatidic acid. The amount of lipid-bound phosphorus (0.05 μmol/mg pollen) did not change significantly during germination, suggesting that the incorporation was mainly due to turnover. *myo*-[2-$^{3}$H]Inositol was incorporated into phospholipids as well as into pectic cell wall polysaccharides. The phospholipids labelled with both $^{3}$H and $^{32}$P were found to be phosphatidylinositol (90% of $^{3}$H-label) and phosphatidylinositol monophosphate. The identity of the latter was established by thin layer chromatography, by anion exchange column chromatography (with immobilized neomycin sulphate as the stationary phase), and by the formation of glycerylphosphoryl inositol phosphate and inositol bisphosphate during alkaline hydrolysis. Pulse–chase experiments suggested that about 40% of the *myo*-inositol moieties in phosphatidylinositol is exchanged during the 4 hr-period of most rapid pollen tube extension. This high figure may be a consequence of membrane flow known to take place during pollen tube elongation, proceeding at the high rate of approximately 1 mm/4 hr. Phosphatidylinositol phospholipase C activity, which could play a role in this turnover, was detected in pollen tube homogenates. Phospholipase activities towards the other major phospholipids were also observed.

## INTRODUCTION

Phytic acid, or *myo*-inositol hexa-*o*-kisphosphate, is a commonly observed storage compound in seeds and pollen [1]. In pollen of *Petunia hybrida*, a dicotyledonous plant, the metabolic fate of this compound has been investigated [2, 3]. Its degradation, which is catalysed by a cytoplasmic phytase [4], results in the release of *myo*-inositol and phosphate. Both constituents are incorporated into phospholipids, while *myo*-inositol is also used for biosynthesis of pectic cell wall polysaccharides [3]. Most of our knowledge of the biosynthesis of pectin from *myo*-inositol has emerged from studies with germinating pollen of a different species, the monocotyledonous *Lilium longiflorum* (for a review, see Loewus and Loewus [5]). Pollen tube elongation in this species occurs at a much higher rate than for *Petunia*. Due to this rapid elongation an intensive metabolism of phospholipids may be expected, because an active membrane flow in addition to cell wall synthesis is required for pollen tube growth. Evidence for membrane flow comes from electron microscopic studies showing a high production of secretory vesicles from the dictyosomes that accumulate at the pollen tube tip and fuse there with the plasma membrane, secreting cell wall precursors [6–8]. In animal cells inositol phospholipids or phosphoinositides are involved in the regulation of numerous secretory events (for a review see Berridge and Irvine [9]), giving rise to the phenomenon known as the 'inositol lipid response' or 'PI response'. The primary event in such a response is the induction by an extracellular stimulus (e.g. hormone, neurotransmitter) of the phospholipase C catalysed cleavage of polyphosphoinositides. In *Petunia* pollen we observed the incorporation of label into phosphatidylinositol, but not into the polyphosphoinositides such as phosphatidylinositol 4-monophosphate or phosphatidylinositol 4,5-bisphosphate, which have not been reported in plant tissues so far.

Because of our interest in both phytate metabolism and the regulation of pollen tube growth, we investigated the metabolic fate of *myo*-[2-$^{3}$H]inositol and [$^{32}$P]orthophosphate in germinating pollen of *Lilium longiflorum* with special emphasis on turnover of these moieties in inositol phospholipids.

## RESULTS

### *Uptake and incorporation of* myo-[2-$^{3}$H]*inositol*

When germinating pollen of *Lilium longiflorum* was incubated in the presence of *myo*-[2-$^{3}$H]inositol, the uptake of tritium from the medium began slowly (Fig. 1a) but the rate increased gradually with time. The $^{3}$H-content of the non-lipid soluble cell material increased linearly with time (Fig. 1b). Anion exchange (formate form, see Chen and Loewus [10]) and paper chromatography in ethyl acetate–pyridine–water (10:6:5) of this fraction showed *myo*-inositol as the only labelled compound at the early stages of incubation, while other

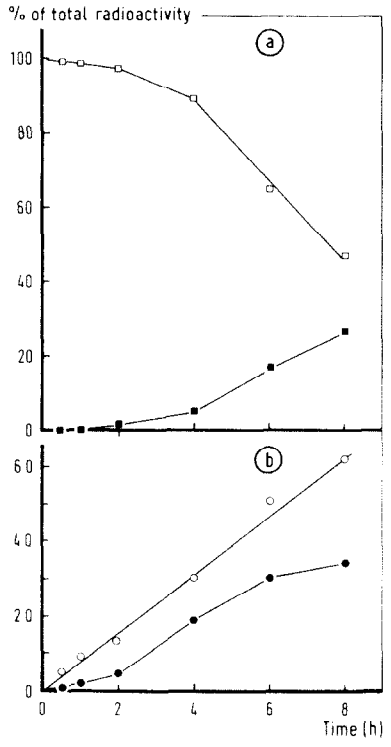



Fig. 1. (a) Uptake of *myo*-[2-$^3$H]inositol (50 $\mu$M) from the medium (□), by *Lilium longiflorum* pollen during 8 hr of germination and incorporation of radioactivity into pectic polysaccharides (■), and (b) into non-lipid, soluble cell contents (○) and lipids (●).

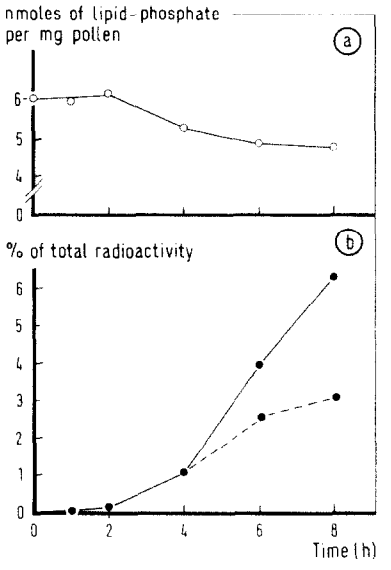



Fig. 2. (a) Content in lipid-bound phosphate (○) and (b) incorporation of [$^{32}$P]orthophosphate into phospholipids (●) in germinating *Lilium longiflorum* pollen during 8 hr of incubation with 50 $\mu$M *myo*-inositol and 110 $\mu$M orthophosphate. The dotted line shows the time course of $^{32}$P-incorporation in a pulse–chase experiment. Here medium $^{32}$P was removed after 4 hr labelling and incubation was continued in unlabelled medium, containing 20 mM *myo*-inositol.

radioactive precursors for pectic cell wall polysaccharides, such as galacturonic acid and arabinose, appeared later. The incorporation into pectic cell wall polysaccharides was slow during the first two hours (Fig. 1a), then the incorporation rate increased and reached a constant level by 4 hr.

In addition to these cell wall related carbohydrates, a considerable part of the tritium was incorporated into the lipid fraction (Fig. 1b). The time course for this incorporation was sigmoidal. At 30 min the amount of lipid-bound tritium was five times higher than in pectic polysaccharides, but during the course of incubation the incorporation into the latter fraction increased faster and was eight-fold that in the lipid fraction after 8 hr.

In the above studies Dickinson's sucrose germination medium was used [11]. Almost identical results were obtained with pentaerythritol medium, which is free of metabolizable sugar [11]. After 8 hr of incubation about 60% of the pollen was germinated.

### *Incorporation of* [$^{32}$P]*orthophosphate in phospholipids*

After a lag period of about 2 hr the rate of incorporation of $^{32}$P into total phospholipid increased up to 4 hr and subsequently remained linear until 8 hr (Fig. 2b). By this time about 6% of the total $^{32}$P was incorporated in the lipid fraction. The amount of lipid-bound phosphate did not increase during germination (Fig. 2a). The uptake of $^{32}$P in non-lipid soluble cell contents was about 7–8 times that in the lipid fraction (data not shown) and followed a similar time course. Incorporation into alcohol-insoluble material was negligible.

TLC of the $^{32}$P-labelled lipid fraction in solvents A–C showed that the majority of the label was in five phospholipids. Phosphatidylcholine and phosphatidylethanolamine each contained more than 30% of total lipid-bound $^{32}$P (Table 1), 13% was found in phosphatidylinositol, 10% in phosphatidylglycerol and 5% in phosphatidic acid. About 3% of the lipid $^{32}$P remained at the origin of the thin layer chromatograms. Phosphatidylserine was not labelled. The distribution of unlabelled phosphate over these phospholipids was similar to that of radioactive $^{32}$P (Table 1) and remained constant during germination. The time course for $^{32}$P-incorporation into the five individual phospholipids was similar to that shown for phosphatidylinositol and phosphatidylethanolamine in Fig. 3, as long as the pollen was heated prior to homogenization.

### *Effects of isotope dilution*

When pollen, germinated for 4 hr in 50 $\mu$m *myo*-[2-$^3$H]inositol was given unlabelled *myo*-inositol to a final concentration of 20 mM, the level of tritium in the lipid fraction immediately started to decrease (Fig. 4b). At 8 hr the radioactivity in this fraction had dropped to about one-half the level that was reached after 4 hr. The radioactivity in the non-lipid soluble cell contents responded to isotope dilution with an initial drop in tritium level (Fig. 4a), and then after 1 hr further, the radioactivity in this fraction remained constant for the rest of the incubation. The incorporation of radioactivity into the pectic polysaccharides continued to increase during

Table 1. Distribution of unlabelled phosphate and of $^{32}P$ in the lipid fraction and the effects of heating germinated pollen, before homogenization in methanol

| Phospholipid | Distribution of unlabelled phosphate in the lipid fraction (heated before homogenization) (%) | Dpm in phospholipid | |
|---|---|---|---|
| | | With heating before homogenization | Without heating before homogenization |
| Phosphatidylethanolamine | 28 | 871 600 (38) | 131 700 (6) |
| Phosphatidylcholine | 41 | 723 900 (31) | 29 800 (1) |
| Phosphatidylinositol | 13 | 301 000 (13) | 212 400 (9) |
| Phosphatidylglycerol | 11 | 230 900 (10) | 157 800 (7) |
| Phosphatidic acid | 6 | 122 400 (5) | 276 800 (12) |
| Phosphatidylmethanol | 0 | 0 | 1 447 200 (62) |
| Origin | 1 | 62 400 (3) | 60 300 (3) |
| Total | 100 | 2 312 200 (100) | 2 316 000 (100) |

*Lilium longiflorum* pollen (25 mg) was incubated for 8 hr in Dickinson's sucrose medium. The percentage of radioactivity from total phospholipid-bound $^{32}P$ is given in parentheses.

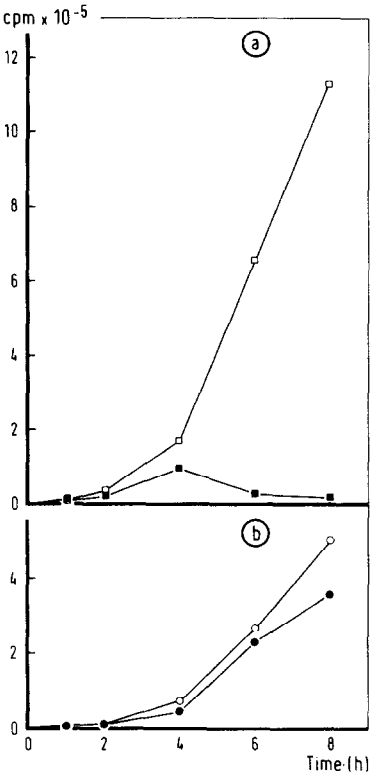



Fig. 3. Incorporation of radioactivity in (a) phosphatidylethanolamine (□) and (b) phosphatidylinositol (○) in germinating *Lilium longiflorum* pollen incubated for 8 hr with 110 $\mu$M [$^{32}P$]orthophosphate and 50 $\mu$M *myo*-inositol. The closed symbols show the time courses for $^{32}P$-levels in these phospholipids from pollen which were not heated (10 min, 100°) prior to homogenization in methanol.

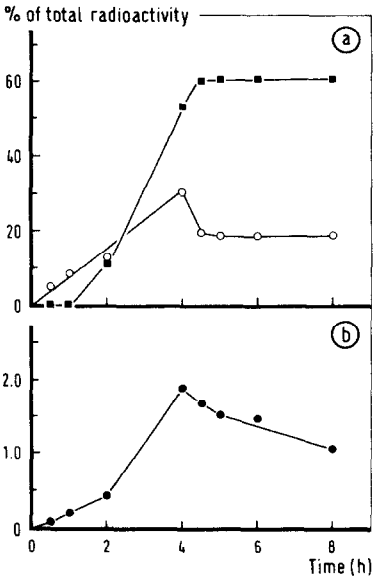



Fig. 4. Effect of radioisotope dilution on the radioactivity in (a) pectic polysaccharides (■) and non-lipid, soluble cell contents (○) and (b) in lipids (●). After 4 hr of germination 4 $\mu$Ci of *myo*-[2-$^{3}H$]inositol was diluted from 50 $\mu$M to 20 mM by the addition of unlabelled *myo*-inositol (100 $\mu$l, 1 M) to 5 ml medium.

the first hour after isotope dilution and thereafter remained at the same level (Fig. 4a).

Similar pulse–chase experiments in which $^{32}P$-prelabelled pollen was incubated in a medium containing unlabelled phosphate, showed that $^{32}P$ was released from the pollen very slowly (data not shown). This is consistent with the observed continued incorporation (at a lower rate) of $^{32}P$ into phospholipid after removal of $^{32}P$ from the medium (Fig. 2b).

### *Identification of phosphatidylinositol monophosphate*

Three methods were used to identify phosphatidylinositol monophosphate in lipid preparations from *Lilium* pollen. The pollen was incubated for 8 hr in the presence of [$^{32}P$]orthophosphate and *myo*-[2-$^{3}H$]inositol. TLC of lipid extracts in solvent D showed both $^{32}P$ and $^{3}H$ label in compounds comigrating with phosphatidylinositol 4-monophosphate ($R_f = 0.45$) and phosphatidylinositol

4,5-bisphosphate ($R_f$ = 0.2) (Fig. 5). The first compound contained about 1% of the lipid-bound $^{32}P$ and 3% of the $^{3}H$. The second compound had 0.5% of the $^{32}P$ and 1% of the $^{3}H$. Most of the $^{3}H$ label was found in phosphatidylinositol ($R_f$ = 0.6), while $^{32}P$ was mainly observed in phosphatidylinositol and other phospholipids (see also Table 1).

Anion exchange column chromatography on neomycin sulphate with methanol solutions containing a discontinuous gradient of ammonium acetate [12], showed that most of the $^{32}P$ and $^{3}H$ label in the lipid fraction eluted with chloroform–methanol–water (3:6:1) containing 150 mM ammonium acetate (Fig. 6a). About 1% was retained and eluted with 600 mM ammonium acetate (Fig. 6a, peak II). At this concentration authentic phosphatidylinositol 4-monophosphate also elutes from the column. TLC of peak II in solvent D showed one labelled compound, which contained both $^{3}H$ and $^{32}P$ and comigrated with phosphatidylinositol 4-monophosphate (Fig. 6b). Subsequent elution of the neomycin anion exchange column with chloroform–methanol–15 M ammonia (3:6:1), which released authentic phosphatidylinositol 4,5-bisphosphate, did not remove any label and so we were not able to verify the presence of this compound.

Alkaline hydrolysis of [$^{32}P$, $^{3}H$]phospholipids from *Lilium* pollen removed all radioactivity from the lipid fraction, indicating the absence of inositol-containing sphingomyelins [13]. Dowex-1 anion exchange chromatography of the methanol–water soluble degradation products [14] with stepwise increasing concentrations of ammonium formate up to 120 mM revealed

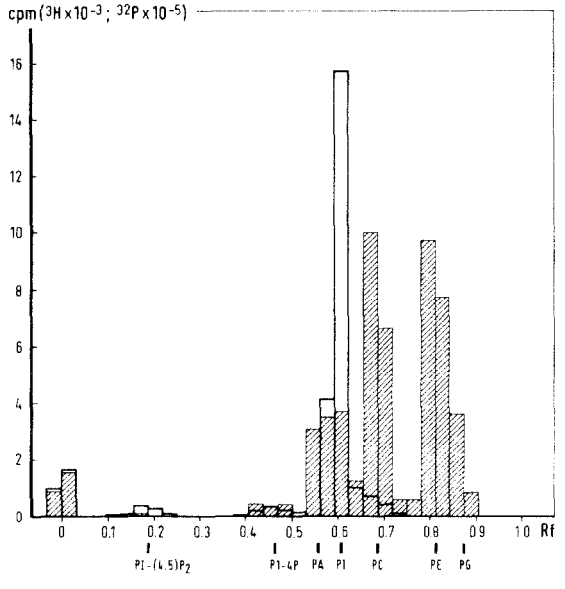
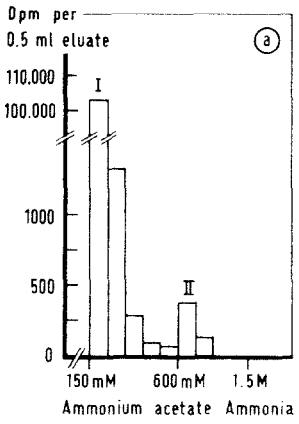



Fig. 5. Thin-layer chromatography of $^{3}H$, $^{32}P$-labelled lipids from *Lilium longiflorum* pollen incubated for 8 hr with 110 $\mu$m [$^{32}P$]orthophosphate (15 $\mu$Ci) and 50 $\mu$M *myo*-[2-$^{3}H$]inositol (0.5 $\mu$Ci). Solvent system: chloroform–methanol–3.3 M ammonia (43:38:12). Heavy lines represent $^{3}H$-counts, striped areas show $^{32}P$-counts. PI-(4,5)$P_2$ = phosphatidylinositol 4,5-bisphosphate; PI-4P = phosphatidylinositol 4-monophosphate; PI = phosphatidylinositol; PA = phosphatidic acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol.



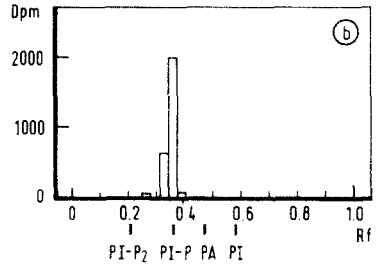




Fig. 6. Identification of phosphatidylinositol monophosphate. (a) Neomycin sulphate anion exchange chromatography of $^{3}H$-labelled lipids. The eluants used were: chloroform–methanol–water (3:6:1), containing 150 mM or 600 mM ammonium acetate, and chloroform methanol 15 M ammonia (3:6:1). I = phosphatidylinositol and II = phosphatidylinositol monophosphate. (b) Thin layer chromatography of fraction II from the neomycin sulphate column with chloroform–methanol–3.3 M ammonia (43:38:12) as the solvent system. Similar results were obtained with $^{32}P$-labelled lipids. PI-$P_2$ = phosphatidylinositol 4,5-bisphosphate; PI-P = phosphatidylinositol 4-monophosphate; PI = phosphatidylinositol; PA = phosphatidic acid.

that most of the radioactivity was in free *myo*-inositol, orthophosphate, inositol phosphate and in glycerylphosphoryl derivatives from phospholipids containing only one secondary phosphate, such as phosphatidylinositol. Further evidence for the presence of phosphatidylinositol monophosphate was obtained by the elution of two double-labelled compounds with higher ammonium formate concentrations. The first one was released with 300 mM ammonium formate, as expected for glycerylphosphoryl inositol phosphate, the deacylation product of phosphatidylinositol monophosphate. The second compound, containing both $^{32}P$ and $^{3}H$, eluted with 400 mM ammonium formate, which releases inositol bisphosphate. No additional radioactivity could be recovered from the column with 500 mM ammonium formate or 0.3 M lithium chloride, which elute glycerylphosphoryl inositol bisphosphate or inositol trisphosphate, respectively. These latter two compounds are possible degradation products of phosphatidylinositol 4,5-bisphosphate.

*Phospholipase activities in germinating pollen*

Omission of the heating step (10 min, 100°) before homogenization of the germinated pollen in methanol did not influence the total $^{32}P$-radioactivity in the lipid fraction (Table 1). The distribution of radioactivity among the various phospholipids was however significantly affected. There was a marked decrease of $^{32}P$-radioactivity in phosphatidylcholine and phosphatidylethanolamine and a much smaller change in phosphatidylglycerol and phosphatidylinositol. The $^{32}P$ from the degraded phospholipids accumulated in phosphatidic acid and in a lipid component, identified as phosphatidylmethanol in solvents A and B [15]. These results are consistent with a phospholipase D-type of cleavage. Figure 3 shows that this phospholipase D activity sharply increased after 4 hr of germination. The time course for this phospholipase D activity towards phosphatidylcholine and phosphatidylglycerol was found to be similar to that shown for phosphatidylethanolamine and phosphatidylinositol, respectively.

To investigate whether or not other types of phospholipase activity are present in *Lilium* pollen tubes, homogenates, suspended in potassium acetate buffer (pH 6.5, 1 mg protein/ml), were incubated with purified $^{32}P$-labelled phospholipids. After incubation for 1 hr at 25° the amount of $^{32}P$ in water-soluble and lipoidal products was determined. Table 2 shows that the inositol phospholipids in particular were sensitive to phospholipase activity in the homogenates as measured by the production of $^{32}P$-labelled water-soluble products. There was considerable enzymatic hydrolysis of phosphatidic acid and phosphatidylethanolamine also, although less than observed for phosphatidylinositol. The enzymatic formation of water-soluble products from $^{32}P$-labelled phosphatidylglycerol and phosphatidylcholine was quite low. TLC of the lipoidal products from incubations showed that phosphatidic acid was observed as reaction product of phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol, indicating degradation by phospholipase D activity as was observed during homogenization in methanol. This was in contrast to the inositol phospholipids, where phosphatidic acid was not observed as a reaction product, suggesting the presence of a phospholipase other than phospholipase D in the homogenate, which is not so active in the presence of methanol. Further investigation suggests that this activity is a phospholipase C activity [16].

Table 2. Percent of radioactivity recovered as water-soluble compounds after incubation (60 min 25°) of untreated and heat-treated (10 min, 100°) pollen tube homogenate with purified $^{32}P$-labelled phospholipids

| | Radioactivity in water-soluble compounds | |
|---|---|---|
| Phospholipid | Untreated homogenate | Heat-treated homogenate |
| | % of total radioactivity | |
| Phosphatidylinositol | 58 | 1 |
| Phosphatidylinositol monophosphate | 64 | 37 |
| Phosphatidylcholine | 6 | 1 |
| Phosphatidylethanolamine | 20 | 1 |
| Phosphatidylglycerol | 11 | 1 |
| Phosphatidic acid | 36 | 2 |

## DISCUSSION

In this paper we show that germinating pollen of *Lilium longiflorum*, cv. Arai, incorporates a significant proportion of *myo*-[2-$^{3}H$]inositol into phospholipid, mainly phosphatidylinositol. The biosynthesis of radioactive cell wall polysaccharides from this precursor follows a similar pattern to that observed by Loewus and Loewus [17] for *Lilium longiflorum* cv. Ace pollen. Indeed, in the early stages of germination, incorporation into phospholipids was even higher than into pectic polysaccharides. The delay in labelling of the latter is probably related to the fact that the *myo*-[2-$^{3}H$]inositol taken up has to be metabolized through the many steps of the *myo*-inositol oxidation pathway [18] before its label can be incorporated into pectins. Phosphatidylinositol can be labelled directly from the tritiated hexitol by reacting with endogenous CDP-diglyceride [19] or by exchange with endogenous, unlabelled phosphatidylinositol [20]. Moreover, pollen tubes do not emerge from the grain during the first hour of incubation and thus the need for newly-synthesized cell wall polysaccharides will be relatively small. Metabolism and synthesis of phosphatidylinositol, as well as other membrane phospholipids, will probably start at the onset of germination.

When pollen tube growth begins at about 2 hr the demand for newly-synthesized pectic polysaccharides and phospholipid biosynthesis or turnover greatly increases, explaining the increased incorporation of [$^{32}P$]orthophosphate and *myo*-[2-$^{3}H$]inositol at this stage. Consistent with earlier observations of Dickinson [11] we have found that the amount of lipid-bound phosphate does not increase during germination of *Lilium longiflorum* pollen. This strongly suggests that the incorporation of both [$^{32}P$]orthophosphate and *myo*-[2-$^{3}H$]inositol is due to turnover of pre-existing phospholipids. The high turnover rate may well be related to the intensive membrane flow which is observed in elongating pollen tubes of not only *Lilium* [6] but also of other plant species [7].

The incorporation of *myo*-[2-$^{3}H$]inositol and [$^{32}P$]orthophosphate into phosphatidylinositol has been observed in other plant species, mostly in studies with isolated organelles [19, 21, 22]. However, the occurrence and the biosynthesis of phosphatidylinositol monophosphate, as described here for *Lilium longiflorum* pollen, has not been reported previously for plant tissue. We did not observe this inositide in *Petunia* pollen [3]. Even though we have some evidence from thin layer chromatography for radioactive phosphatidylinositol bisphosphate, this observation needs further investigation. The chemical lability of this compound was encountered by several authors [2], while the high activity of phosphatases in plant extracts may make its isolation more difficult.

In germinating *Lilium* pollen phosphatidylcholine and phosphatidyl ethanolamine together accounted for about 70% of the lipid-bound phosphate. A similar proportion was observed for the incorporation of [$^{32}P$]orthophosphate. The predominance of these two phospholipids is usually observed in plant tissues [21], as is the distribution over the other major phospholipids. However, in contrast to our observations with *Petunia* pollen [2, 3] the present

study shows that *Lilium* pollen also contains phosphatidylglycerol.

Phospholipid turnover requires both degradation of existing pools and resynthesis. Biosynthesis of phospholipids has been demonstrated by the incorporation of [$^{32}$P]orthophosphate and *myo*-[2-$^{3}$H]inositol. As these are hydrolysis products of phytic acid, this reserve compound of pollen and seeds could play an important role for phospholipid biosynthesis in germinating pollen. Active enzymatic breakdown of phospholipids has also been observed. Besides degradation of [$^{32}$P]phosphatidylinositol by phospholipase C activity [16], [$^{32}$P]phosphatidic acid was also enzymatically hydrolysed upon incubation with pollen tube homogenates, probably by a phosphatidate phosphatase. Phospholipase D activity is structurally excluded for this phospholipid and phospholipase A activity was not detected for any of the major phospholipids, since lysoderivatives were not observed. High phospholipase D activity was observed for phosphatidylethanolamine and phosphatidylcholine. The former was apparently also degraded by a phospholipase C-type cleavage, as incubation with pollen tube homogenates released $^{32}$P-labelled water-soluble products.

The detection in germinating pollen of phosphatidylinositol phospholipase C activity [16] in addition to the occurrence of phosphatidylinositol monophosphate, suggests that some of the components at least, necessary for the so-called 'inositol lipid response' [9] are present in pollen. This response appears to be a plasma membrane phenomenon associated with stimuli of many kinds. Because of the possible importance of an inositol lipid response in pollen–pistil interactions we will investigate this possibility further.

It can be calculated from our pulse–chase experiments (Fig. 4b) that the decrease in label in phosphatidylinositol between 4 and 8 hr represents an average net turnover of 40% of the total phosphatidylinositol content of *Lilium* pollen (a total of 6 nmol/mg pollen). Similar pulse–chase experiments with *Petunia hybrida* pollen indicate an average turnover of 50% of the *myo*-inositol moieties in phosphatidylinositol between 2 and 4 hr of germination [3]. As pointed out previously, a high turnover rate may be the reflection of membrane flow and membrane recycling, known to take place during pollen tube growth, which in *Lilium longiflorum* is extending at the very high rate of 0.25 mm/hr [17].

## EXPERIMENTAL

*Chemicals.* All chemicals were reagent grade. *myo*-[2-$^{3}$H]Inositol and [$^{32}$P]orthophosphate were purchased from Amersham International Inc. and diluted with unlabelled carrier to the desired sp. act. (see below).

*Plant material.* Pollen was collected from dehisced anthers of *Lilium longiflorum* (cv. Arai) plants, grown under greenhouse conditions and stored at −20°. Before incubation pollen was suspended (5 mg/ml) in Dickinson's sucrose or pentaerythritol medium [11] the latter being free of metabolizable sugar.

*Incubation and fractionation of germinated pollen.* After addition of radioactive substrate, *myo*-[2-$^{3}$H]inositol (50 μM, 0.8 μCi/ml) and/or [$^{32}$P]orthophosphate (110 μM, 5 μCi/ml), 5 ml portions of the pollen suspension were placed in 25 ml Erlenmeyer flasks and incubated at 25° on a gyratory shaker. In pulse–chase experiments radioactive *myo*-[2-$^{3}$H]inositol was diluted out after 4 hr of incubation by the addition of 100 μl unlabelled *myo*-inositol (final concn 20 mM). Adhering label was removed from the germinated pollen by washing twice with 1.0 ml fresh medium. Endogenous phospholipases were inactivated by heating for 10 min at 100° (boiling water bath). If indicated this heating step was omitted. Germinated pollen was homogenized in 1.0 ml MeOH with an all-glass potter homogenizer, 2.0 ml $CHCl_3$ and 0.9 ml $H_2O$ were added and the suspension was thoroughly mixed. The homogenate was fractionated into non-lipid, soluble cell contents, lipids and pectic cell wall polysaccharides as described previously [3]. Both aq. phase and interphase were extracted once more with 2.0 ml $CHCl_3$ after addition of 0.1 ml conc. HCl to assure complete recovery of phospholipids in the organic phase [12]. The two organic phases were combined and referred to as 'the lipid fraction'.

Insoluble interphase material was separated from the aq. phase and washed × 3 with 2.0 ml cold 80% EtOH. The radioactivity in the alcohol-insoluble material represents pectic polysaccharides, predominantly consisting of labelled galacturonic acid and arabinose with glucose and xylose as minor radioactive components [10]. The aq. phase was combined with the EtOH washes and is referred to as 'alcohol-soluble, non-lipid cell contents'.

*Phospholipase assays with pollen tube homogenates.* Pollen tube homogenates were prepared from *Lilium* pollen which germinated in sucrose medium for 4 hr as described above. At this stage phosphatidylinositol phospholipase activity was maximal [16]. After two washes with 2 ml 0.1 M KOAc buffer (pH 6.5), containing 2 mM $CaCl_2$, pollen tubes were homogenized in 1 ml of the same buffer with an all glass potter homogenizer. Cell debris was removed by centrifugation (5 min 1200 *g*), the supernatant diluted to 1 mg protein/ml, dialysed for 24 hr against the above-mentioned buffer and stored at −20°.

$^{32}$P-Labelled lipid fractions, containing the phospholipids used as substrates for the assays, were obtained from 25 mg *Lilium* pollen, which germinated for 8 hr in sucrose medium in the presence of 50 μCi [$^{32}$P]orthophosphate. [$^{32}$P]Phospholipids were purified by TLC in solvent B (see 'analytical procedures') except for the purification of phosphatidylinositol monophosphate, for which solvent D was used. After localization with reference compounds and radioautography the phospholipids were scraped off together with the silica gel, eluted with 4 ml $CHCl_3$–MeOH (1:1) and stored at −20°.

For phospholipase assays 50 μl of these purified [$^{32}$P]phospholipids (0.5–3 nmol) were evaporated to dryness under a stream of $N_2$. Incubations were carried out at 25° for 1 hr and started by the addition of 0.9 ml pollen tube homogenate (0.9 mg protein). Reactions were stopped by heating for 10 min at 100°. Radioactive lipoidal and water-soluble compounds were separated as described before [3]. Phospholipase activities were determined from the distribution of radioactivity in lipoidal and water-soluble compounds.

*Analytical procedures.* TLC of phospholipids was performed on activated (10 min, 110°) silica gel 60 plates (Merck no. 5721) in the following solvent systems: $CHCl_3$–MeOH–$H_2O$ (62:25:4) (solvent A); $CHCl_3$–MeOH–HOAC (60:25:8) (solvent B) or $CHCl_3$–MeOH–HOAC–$H_2O$ (50:28:4:8) (solvent C). Phosphatidylinositol monophosphate and phosphatidylinositol bisphosphate were identified in $CHCl_3$–MeOH–3.3 M $NH_3$ (43:38:12) (solvent D) [12]. Authentic phospholipids, obtained from Sigma, served as reference compounds.

The occurrence of labelled polyphosphoinositides was also investigated by anion exchange chromatography on neomycin sulphate columns (1 ml total vol. contained in a Pasteur pipette) according to Schacht [23] and by alkaline hydrolysis of the lipid fraction with subsequent analysis on Dowex-1 (formate form, 1 ml) anion exchange chromatography of the labelled, water-soluble degradation products [14]. These were released from the

column by subsequent elution with 30 ml aq. solns, containing ammonium formate in the following concns: 0 mM, 60 mM, 120 mM, 200 mM, 300 mM, 400 mM and 500 mM. Finally the column was eluted with 30 ml 0.3 M LiCl.

Radioactive analysis was carried out in a Philips PW 4540 liquid scintillation analyser with 5 ml Tritosol [24] as a scintillation cocktail. Alcohol-insoluble material was first treated with 2 M $CF_3CO_2H$ (40 min, 125°), which dissolves more than 99% of the radioactivity, before liquid scintillation analysis was carried out as described above.

Protein was assayed according to Bradford [25]. Lipid-bound phosphorus was determined according to Rouser *et al.* [26].

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## NOTE ADDED IN PROOF

After the revised version of this article was submitted the occurrence of phosphorylated inositol lipid was reported by Boss, W. F. and Massel, M. O. (1985) *Biochem. Biophys. Res. Commun.* **132**, 1018 and by Heim, S. and Wagner, K. G. (1986) *Biochem. Biophys. Res. Commun.* **134**, 1175.