Phosphatidylinositol Cycle Metabolites in *Samanea saman* Pulvini[1]

M. J. Morse, Richard C. Crain, and Ruth L. Satter*
Department of Molecular and Cell Biology, U-42, The University of Connecticut, Storrs, Connecticut
06268

ABSTRACT

The major metabolites of the phosphatidylinositol cycle from extracts of [^{32}PO$_4$]- and [^{3}H]-inositol-labeled *Samanea saman* pulvini were separated. The membrane localized phosphoinositides were separated by thin layer chromatography, identified by comparison with purified lipid standards, and quantitated based on incorporation of radiolabel. The ratio of radioactivity in phosphatidylinositol:phosphatidylinositol 4-phosphate: phosphatidylinositol 4,5-bisphosphate is about 32:8:1. The aqueous inositol phosphates were separated by anion exchange chromatography using conventional liquid chromatography and by high performance liquid chromatography (HPLC) and were identified by comparison with standards. Analysis by HPLC reveals that ^{32}P-labeled pulvini have inositol 1-phosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate that co-migrate with red blood cell inositol phosphates, but ^{3}H-inositol-labeled pulvini appear to have a variant profile.

Leaflet movements in the nyctinastic legume *Samanea saman* are driven by a circadian clock that can be reset by red light absorbed by phytochrome (23), or by blue light absorbed by an unidentified pigment (21). Knowledge of the transduction steps that couple light absorption to cellular responses are of great interest, both for understanding biochemical events that follow excitation, and for probing light-clock interaction.

We are investigating the possibility that the hydrolysis of membrane localized phosphoinositides accompanied by an increase in cytosolic free calcium mediates phototransduction in *Samanea* motor cells. That phosphatidylinositol turnover may be involved is suggested by the following: (a) in animal tissues, PI[2] turnover has been demonstrated to mediate light- and hormone-activated cellular responses that depend upon calcium mobilization (1, 8, 20); (b) in plant cells, the lipid components of the PI cycle have been identified (2); (c) hormone stimulated turnover of PI (15) and inositol trisphosphate-stimulated release of Ca^{2+} (7, 18) have been reported in soybean microsomes and carrot protoplasts; (d) increased cytosolic free Ca^{2+} regulates a myriad of activities in plant cells (11, 22) and may be required

for phytochrome-mediated responses (4, 11, 19) and rhythmic phase shifting (10).

The PI cycle as elucidated in animal tissues (Fig. 1) involves the following series of reactions (1, 6, 16, 17): external signals such as light, hormones, or neurotransmitters interact with receptors on the plasma membrane. These interactions stimulate G protein to bind GTP and then to activate a phosphodiesterase (phospholipase C). Phospholipase C then hydrolyzes PIP$_2$, yielding diacylglycerol and IP$_3$. Each product activates a well-defined biochemical pathway. Diacylglycerol activates protein phosphorylation via protein kinase C (16, 17). IP$_3$ is released into the cytoplasm, where it functions as a second messenger that mobilizes intracellular Ca^{2+} from the ER (1). It is not yet known whether the cycle functions similarly in plant cells.

In the present study, we describe the separation of phosphoinositides and inositol phosphates from *Samanea* pulvinar extracts.

MATERIALS AND METHODS

Terminal secondary pulvini, each attached to a 3 cm section of rachilla, were excised from *Samanea saman* plants grown with 16 h light:8 h dark cycles (cool-white fluorescent light, 200 μmol m^{-2} s^{-1}) at 26°C ± 1.5°C (21). All manipulations that took place during the usual 8 h dark period were performed with a dim green "safelight" as the only illumination. The pulvini were cut at h 4.5 of the dark period and the cut end of the rachilla was incubated in water for 19.5 h, *i.e.* for the remaining 3.5 h of the dark period followed by the 16 h light period. At the beginning of the next dark period, the water was replaced by a solution of either ^{32}P-orthophosphate or myo-2-^{3}H(N)-inositol (each 60 μCi/ml) for 6 h. At the end of the incubation (h 6 of the dark period), the pulvinus was immediately frozen in liquid N$_2$. The frozen tissue was pulverized with a mortar and pestle on dry ice. Further metabolic reactions were stopped by addition of 2 ml chloroform:methanol:3 N HCl (6:6:1 v/v), and the extract was washed according to the method of Folch *et al.* (9). The organic phase was taken to dryness under N$_2$, redissolved in chloroform:methanol (2:1) and applied to silica gel 60 plates with or without oxalate (E. Merck, Darmstedt, Germany) or to oxalate impregnated silica gel HL plates (Analtech, Newark, DE). Oxalate complexes residual Ca^{2+}, which has been reported to alter the chromatographic mobility of PIP and PIP$_2$ (2, 13). However, we found adequate separation of all inositol lipids on silica gel 60 plates without oxalate. The plates were developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, v/v) according to Jolles *et al.* (14). ^{32}P- or ^{3}H-labeled phospholipids were detected by autoradiography; plates were sprayed with En^{3}Hance (New England Nuclear) and developed on Kodak X-Omat x-ray film at −70°C (^{32}P, 0.5 d; ^{3}H, 7–14 d). Identification of radiolabeled phospholipid was made by comparison with

[2] Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; IP, inositol 1-phosphate; IP$_2$, inositol 1,4-bisphosphate; IP$_3$, inositol 1,4,5-trisphosphate.
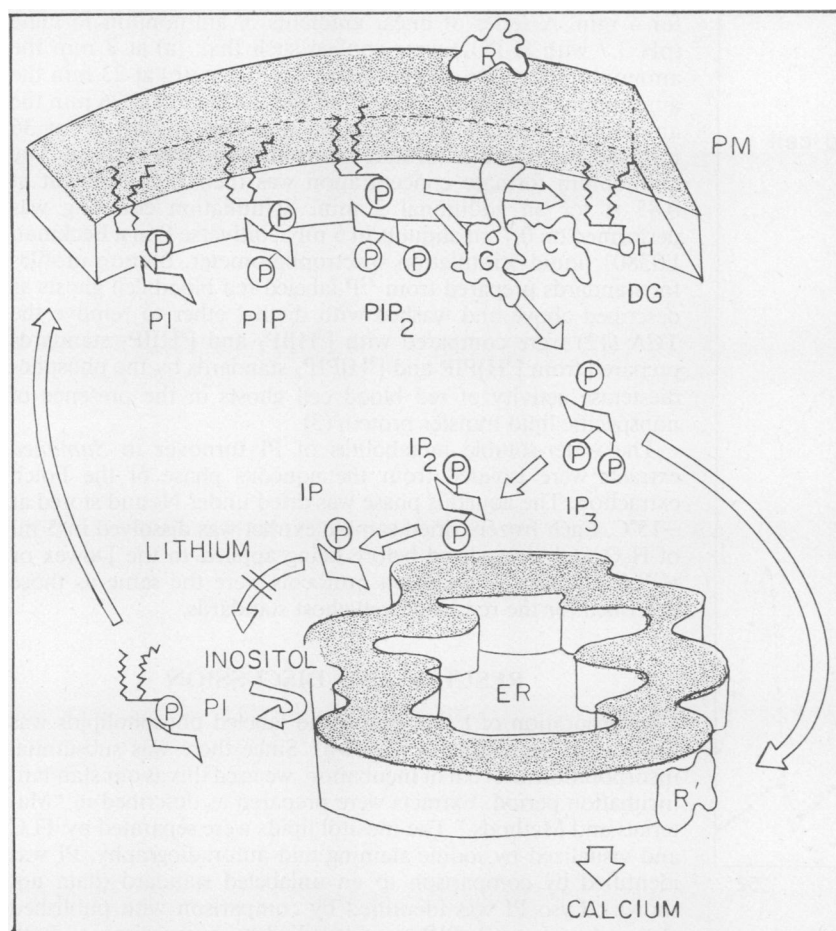
FIG. 1. Schematic representation of phosphoinositide metabolism, as elucidated in animal tissues. A phosphodiesterase (phospholipase C) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to yield diacylglycerol and inositol 1,4,5-trisphosphate (IP₃). IP₃ binds to a receptor (R′) on the ER and stimulates Ca^{2+} efflux. IP₃ is rapidly hydrolyzed by phosphodiesterases, first to inositol 4-phosphate (IP₂), then to IP, and finally to inositol. This inositol, together with inositol taken up by the cell and inositol provided by *de novo* synthesis, is used for the resynthesis of PI, which takes place in the ER. PI is incorporated into the plasma membrane, and subsequently phosphorylated by kinases to PIP and PIP₂. Abbreviations: R, receptor for agonist stimulated PI turnover; G, G protein; C, phospholipase C; R′, IP₃ receptor; ER, endoplasmic reticulum; PM, plasma membrane. Modification of the model of Berridge and Irvine (1).

authentic standards of PI, PIP (Sigma Chemical Co), ³H-PIP, and ³H-PIP₂ (New England Nuclear). Labeled bands were scraped and radioactivity was quantitated by liquid scintillation counting.

Water soluble standards for IP, IP₂, and IP₃ were generated from ³²P-labeled red blood cell ghosts by activation of an endogenous phosphodiesterase that hydrolyzes membrane bound phosphoinositides (5). Briefly, 0.5 ml of red blood cell ghosts were incubated for 30 min at 37°C in 1 ml Hepes buffer containing 2 mM Ca^{2+}. The reaction was stopped by addition of 1 ml 10% TCA and chilled. The precipitate was sedimented and the supernatant was adjusted to a total volume of 5 ml with water and to neutral pH by dropwise addition of 1 N NaOH. The labeled PI hydrolysis products IP, IP₂, and IP₃ were separated by anion exchange chromatography using 1 ml of Dowex 1-10X (5) and/or by HPLC (12) using a Partisil SAX column (Whatman Inc). For Dowex anion exchange chromatography, the column was eluted with 10 ml H₂O followed by 8 ml of each of the following: (a) 5 mM sodium tetraborate, 60 mM sodium formate; (b) 0.2 M ammonium formate, 0.1 M formic acid; (c) 0.4 M ammonium formate, 0.1 M formic acid; (d) 1 M ammonium formate, 0.1 M formic acid. The eluants were collected as 1 min fractions (approximately 0.85 ml) and radioactivity quantitated by liquid scintillation counting. The eluant peaks of radioactivity were used as standards. For HPLC, the elution gradient was a modification of that described by Berridge *et al.* (12). The flow was maintained at 2.5 ml/min and fractions were collected at 30 s intervals. The sample was applied in water and eluted with water
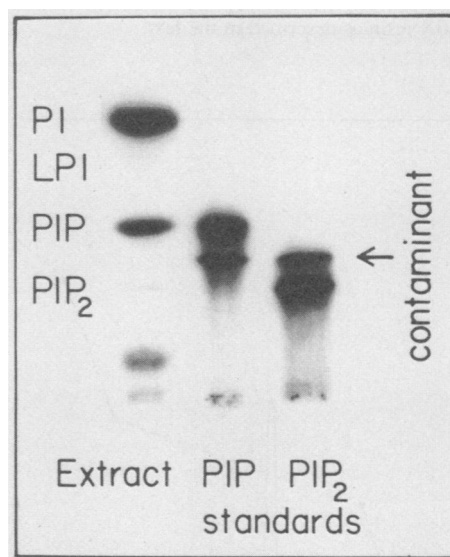

FIG. 2. Autoradiograph of the inositol lipids PI, PIP, and PIP₂, separated by TLC. Total phospholipid from a *Samanea* pulvinus prelabeled with [³H]myo-inositol for 6 h (60 μCi/ml) was applied to a silica gel 60 plate, developed, and autoradiographed as described in the text. Migration of the radiolabeled bands was compared to that for [³H]PIP and [³H]PIP₂ standards.
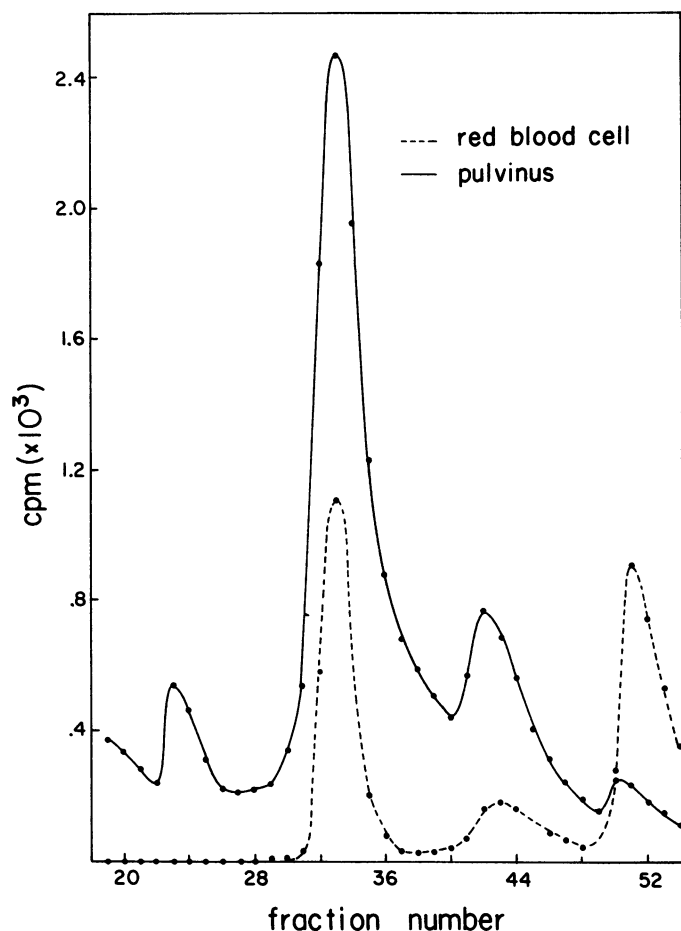
FIG. 3. Separation of the water-soluble products of phosphoinositide metabolism from *Samanea* motor tissue extracts and from red blood cell ghost extracts. *Samanea* motor tissue and red blood cell ghosts were labeled, extracted, and separated by anion exchange chromatography on a Dowex 1-10X resin as described in the text.

for 4 min. A series of linear gradients of ammonium formate (pH 3.7 with H_3PO_4) were applied such that: (a) at 8 min the ammonium formate concentration was 0.3 M, (b) at 23 min the ammonium formate concentration was 0.43 M, (c) at 26 min the ammonium formate concentration was 0.64 M, and (d) at 36 min the ammonium formate concentration was 0.85 M. The ammonium formate concentration was then kept constant at 0.85 M for an additional 2 min. Scintillation counting was performed on 0.7 ml aliquots in 5 ml Scintiverse E in a Beckman LS3801 liquid scintillation spectrophotometer. Elution profiles for standards prepared from ^{32}P-labeled red blood cell ghosts as described above and washed with diethyl ether to remove the TCA (12) were compared with [^{3}H]IP$_2$ and [^{3}H]IP$_3$ standards prepared from [^{3}H]PIP and [^{3}H]PIP$_2$ standards by the phosphodiesterase activity of red blood cell ghosts in the presence of nonspecific lipid transfer protein (3).

The water-soluble metabolites of PI turnover in *Samanea* extracts were isolated from the aqueous phase of the Folch extraction. The aqueous phase was dried under N$_2$ and stored at −15°C. Each frozen, dried sample extract was dissolved in 5 ml of H$_2$O and neutralized before being applied to the Dowex or HPLC column. The elution protocols were the same as those described for the red blood cell ghost standards.

RESULTS AND DISCUSSION

Incorporation of [^{3}H]inositol into labeled phospholipids was linear with time (data not shown). Since there was substantial incorporation after 6 h of incubation, we used this as our standard incubation period. Extracts were prepared as described in "Materials and Methods." The inositol lipids were separated by TLC and visualized by iodine staining and autoradiography. PI was identified by comparison to an unlabeled standard (data not shown); Lyso PI was identified by comparison with published chromatograms (2); PIP was identified by comparison to both unlabeled PIP (data not shown) and radiolabeled standard (Fig. 2); PIP$_2$ was identified by comparison to radiolabeled standard (Fig. 2). Standard radiolabeled PIP and PIP$_2$ both show a contaminating component that migrates at an R$_F$ intermediate between the two. This component does not migrate with any major
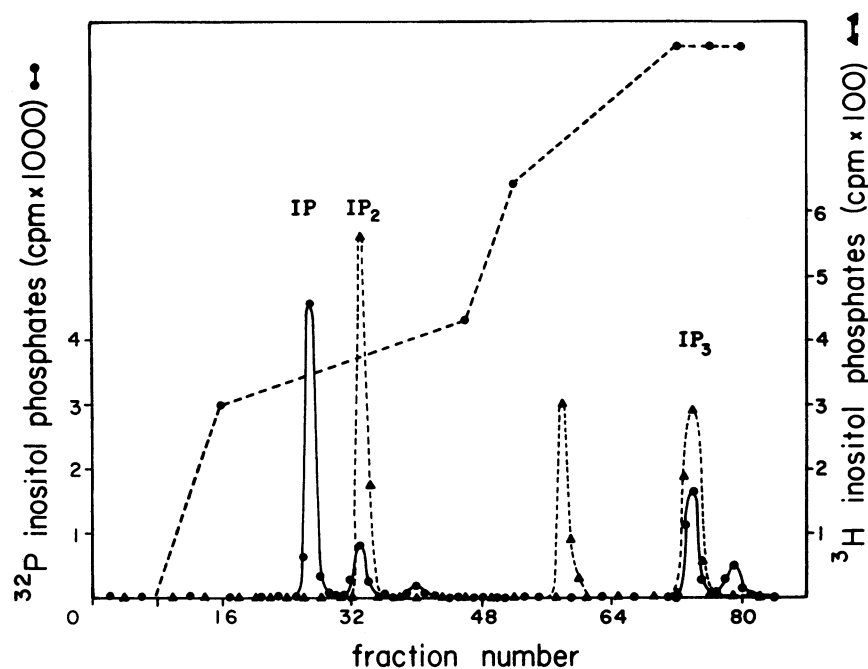

FIG. 4. Comparison of ^{32}P-red blood cell inositol phosphates with [^{3}H]inositol phosphate standards. ^{3}H-labeled polyphosphoinositide standards were used to generate inositol phosphate standards (▲−−−▲) and separated by HPLC for comparison to ^{32}P-labeled red blood cell standards (●──●). The gradient of ammonium formate used in Figures 4 to 6 is indicated by the dashed line.

lipid band from the labeled pulvini. Furthermore, when the lipid contaminant is hydrolyzed by red blood cell phosphodiesterase, the water soluble product elutes between IP$_2$ and IP$_3$ using HPLC (see below). Quantitation of radioactivity in the chromatogram revealed that 75.9 ± 3.4% (mean ± SE, $n = 4$) of the label was present in PI, 1.3 ± 0.3% was present in lyso PI, 19.5 ± 2.7% was present in PIP, and 2.4 ± 0.3% was present in PIP$_2$.

We identified the phosphoinositide water-soluble metabolites (IP, IP$_2$, and IP$_3$) from the aqueous phase from extracted *Samanea* pulvini by comparison with red blood cell standards. Figure 3 shows the Dowex anion exchange separation of ^{3}H inositol-labeled water soluble metabolites from pulvinar extracts and from ^{32}P-labeled red blood cell ghosts. Based on published profiles (5) and similarity in eluant positions for red blood cell and pulvinar extracts, we initially identified the peaks, in order of elution, as IP (fraction 30–40), IP$_2$ (fraction 40–48), and IP$_3$ (fraction 48–54). However, the red blood cell and pulvinar peaks did not coincide identically. To obtain better resolution, we
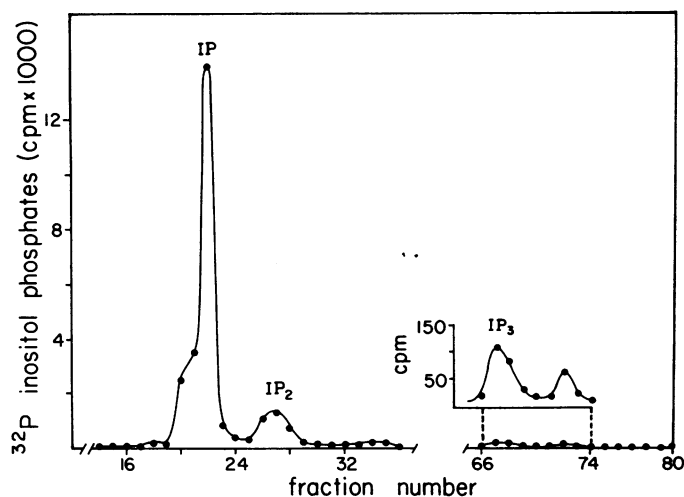
performed additional separations of the inositol phosphates by HPLC.

HPLC separation of red blood cell inositol phosphates is shown in Figure 4. Four major peaks of radioactivity are apparent. Based on comparison with separations reported by Heslop *et al.* (12) and with the inositol phosphate composition reported for red cell extracts (5), the peaks are identified, in order of elution, as IP, IP$_2$, IP$_3$ (Fig. 4). In agreement with this identification, [^{3}H] IP$_2$ and [^{3}H]IP$_3$ standards formed by hydrolysis of exogenously supplied [^{3}H]PIP and [^{3}H]PIP$_2$ standards (as described in "Materials and Methods") coelute with the red blood cell standards. The major peak eluting between IP$_2$ and IP$_3$ reflects the major contaminant in the lipid standards from which these were derived (Fig. 2).

We also separated both ^{32}P-labeled and ^{3}H-labeled pulvinar inositol phosphates by HPLC. Figure 5 shows the separation of water soluble extracts from ^{32}P-labeled pulvini. As with red blood cell extracts (Fig. 4), four peaks were apparent and the first three were identified as IP, IP$_2$, IP$_3$. Careful examination of the elution profile in Figure 5 shows that the entire profile eluted 4 to 5 fractions more rapidly than did the red blood cell standards. We attribute the slightly altered elution positions of the two samples to the different pH and ionic compositions of the extracts. This was verified by our finding that coinjection of equal amounts (cpm) of the two samples resulted in single peaks of [^{32}P]IP and [^{32}P]IP$_2$ (data not shown).

Water soluble extracts of pulvini labeled for 6 h with [^{3}H] inositol were also separated by HPLC and the profiles of the eluted inositol phosphates compared with [^{32}P]inositol phosphates from either red blood cell ghosts or pulvini. Figure 6 reveals three peaks of radioactivity for [^{3}H]inositol labeled pulvini; the first two elute before red blood cell IP while the third comigrates with red blood cell IP$_2$. These results are not due to different extraction conditions since coextraction of red blood cell ghosts and pulvini yields the same profile. Furthermore, we have observed similar profiles when ^{32}P-labeled pulvinar extracts and ^{3}H-labeled pulvinar extracts were co-injected, *i.e.* two tritium peaks elute before [^{32}P]IP. We are currently examining the nature of the "inositol phosphates" and the reactions that produce them.

In summary, we have presented evidence that *Samanea saman* motor cells have all of the phospholipids and lipid metabolites of the PI cycle and we have described the separation and preliminary identification of these metabolites. We will now determine

Fig. 5. Separation of ^{32}P-labeled pulvinar inositol phosphates by HPLC. *Samanea* pulvini labeled with ^{32}PO$_4$ were extracted and the water soluble metabolites were separated by HPLC as described in "Materials and Methods."
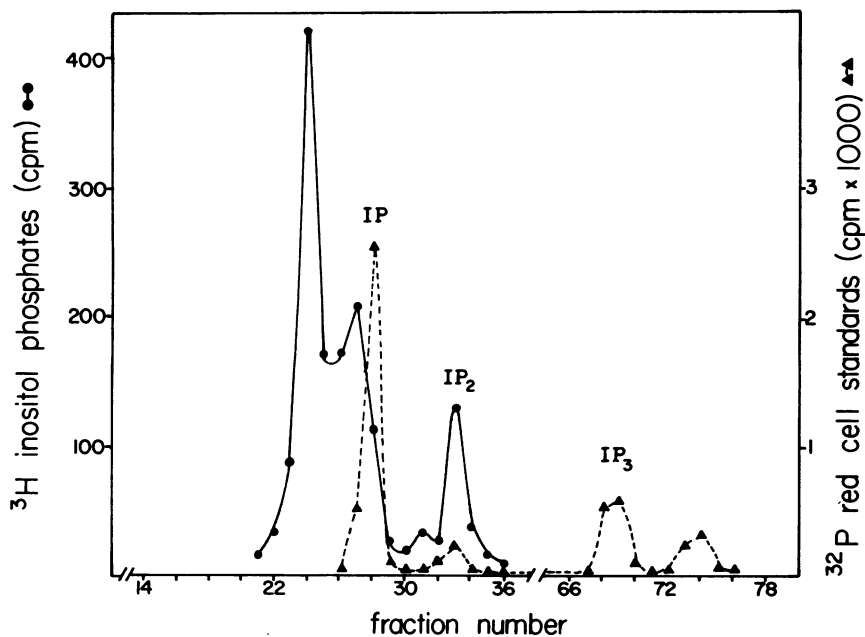

Fig. 6. Separation of labeled pulvinar and red blood cell inositol phosphates by HPLC. *Samanea* pulvini were labeled with ^{3}H-inositol and were extracted as described in "Materials and Methods." The aqueous phase was dried, reconstituted in water and neutralized, the pulvinar fraction (●——●) was then mixed with the red blood cell fraction (▲---▲) and the inositol phosphates were separated by HPLC.

whether photic stimuli are transduced by a mechanism involving PI turnover.

Note Added in Proof. A further refinement of the HPLC gradient resolves IP, IP$_2$, and IP$_3$ in ^{3}H-labeled *Samanea* and disclosed a considerably more complex profile for ^{32}P-labeled material (G Coté, MJ Morse, RC Crain, RL Satter, unpublished data).

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