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Metabolic Relations of Inositol 3,4,5,6-Tetrakisphosphate Revealed by Cell Permeabilization. Identification of Inositol 3,4,5,6-Tetrakisphosphate 1-Kinase and Inositol 3,4,5,6-Tetrakisphosphate Phosphatase Activities in Mesophyll Cells[1]

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Using a permeabilization strategy to introduce Ins(3,4,5,6) P_4 into mesophyll protoplasts of *Commelina communis*, we have identified Ins(3,4,5,6) P_4 1-kinase activity in mesophyll cells. Multiple $InsP_3$ isomers were identified in *Spirodela polyrhiza* and Arabidopsis. Only two of these, Ins(1,2,3) P_3 and Ins(3,4,6) P_3, have previously been identified in plants and only in monocots. The isomers detected in *S. polyrhiza* included D- and/or L-Ins(3,4,5) P_3, D- and/or L-Ins(3,5,6) P_3, and D- and/or L-Ins(2,4,5) P_3. Ins(1,4,5) P_3, if present, was only a tiny fraction of total $InsP_3$ species. We have also identified inositol polyphosphate phosphatase activities, Ins(3,4,5,6) P_4 6-phosphatase and Ins(3,4,5, 6) P_4 4-phosphatase, whose action on endogenous inositol polyphosphates explains the presence of D- and/or L-Ins(3,4,5) P_3 and D- and/or L-Ins(3,5,6) P_3 in mesophyll cells. Inositol trisphosphates identified in Arabidopsis include Ins(1,2,3) P_3 and D- and/or L-Ins(3,4,6) P_3, suggesting that dicots may share pathways of $InsP_6$ biosynthesis and breakdown in common with monocots.

Perhaps the single most distinctive feature of plant inositol phosphate metabolism is the accumulation of inositol hexakisphosphate ($InsP_6$)[2] to levels up to several percent of dry weight in seed or storage tissues (Raboy and Dickinson, 1987) and in vegetative tissues to levels that are likely to be in excess of other inositol phosphates. It is likely that the inositol phosphates commonly found in plants are not related to the signaling molecule Ins(1,4,5) P_3, but are intermediates of the pathways of $InsP_6$ synthesis and breakdown.

We have described a number of inositol phosphates in the duckweed *Spirodela polyrhiza* (Brearley and Hanke, 1996a) and in barley aleurone tissue (Brearley and Hanke, 1996c). $InsP_4$ and $InsP_5$ species have been identified in mung bean (Stephens, 1990; Stephens et al., 1991) and soybean (Phillippy et al., 1994). Metabolic evidence (Brearley and Hanke, 1996b) suggests that some of those identified in *S. polyrhiza* are intermediates in $InsP_6$ biosynthesis. However, as there is some uncertainty surrounding the order of addition of the 1- and 5-Ps (fourth and fifth in the sequence proposed) to the inositol moiety of $InsP_6$, the validity of the proposed order of the metabolic sequence relies heavily on the nature of the inositol phosphates identified, and, paradoxically, on those present but not yet described. The identification of enzyme activities that phosphorylate endogenous inositol phosphates to products higher in the sequence is of crucial importance, therefore, in distinguishing between possible pathways. Furthermore, as the pathway described in plants differs from that described in *Dictyostelium discoideum* (Stephens and Irvine, 1990) (notwithstanding a report of an alternative nuclear pathway in this organism; Van der Kaay et al., 1995), while that in animals is unclear, the nature of the pathway operating in plants assumes greater significance because it may shed light on the pathways operating in other kingdoms.

Insofar as the patterns of isomers detected in plants are atypical of animal cells and may be indicative of functions for these compounds specific to plants, we have also set out to identify the range of $InsP_3$ species present in two experimental systems: frond tissue of the aquatic monocotyledonous plant *S. polyrhiza* (mesophyll cells predominantly) and root suspension cultures of the dicotyledonous plant Arabidopsis, which as a model experimental system in plant molecular genetics merits attention.

MATERIALS AND METHODS

Plant Material

Spirodela polyrhiza L. plants were labeled with *myo*-[2-^{3}H]inositol (21 Ci/mmol, Amersham International, Buckinghamshire, UK) as described previously (Brearley and Hanke, 1996a). Root cell suspension cultures of Arabidopsis (ecotype Landsberg *erecta*) were obtained from Paul Duprée of the Department of Biochemistry, University of Cambridge. Stock cultures were maintained at 25°C on an orbital shaker in Gamborg's B5 medium (Sigma G-5893, Sigma Chemical, Poole, Dorset, UK) and further supple-

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[2] Inositol phosphates from biological sources, in which the ratio of enantiomers is undefined, are given the prefix D- and/or L-; all other isomers, except *meso* compounds, are assigned as D-isomers except where specifically indicated in the text.

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mented with 20 g/L Glc, 50 mg/L kinetin, 0.5 mg/L 2,4-dichlorophenoxyacetic acid, and 0.5 g/L 2-(N-morpholino)-ethanesulfonic acid (MES), pH 5.7 (22°C). Labeled cultures were initiated by the transfer of a small spatula load of cells from a 7-d-old stock culture to labeling medium comprising Gamborg's B5 basal salt mixture (Sigma G-5768) supplemented as above but also containing 1 mg/L nicotinic acid, 1 mg/L pyridoximine hydrochloride, and 10 mg/L thiamine hydrochloride. Cells 0.26 to 0.34 g fresh weight were removed from a subculture of cells labeled for 4 d in 10 mL of medium containing 20 μCi of *myo*-[2-^{3}H]inositol. *Commelina communis* plants were grown from seed. Seed was originally obtained from Prof. T.A. Mansfield, University of Lancaster, UK.

Tissue Extraction

Labeled *S. polyrhiza* tissue and Arabidopsis suspension cultures were extracted with perchloric acid, neutralized and eluted from a strong anion-exchange HPLC column (Partisphere SAX, Whatman International, Maidstone, UK) with gradients of NaH_2PO_4. Peak fractions were desalted on Dowex AG1 X8 resin (formate form) columns (Brearley and Hanke, 1996a).

Erythrocyte Ghost Treatment of Inositol Phosphates

Desalted inositol phosphates were treated with erythrocyte ghosts under ionic conditions, 12.5 mM-HEPES, pH 7, 10 mM-$MgCl_2$, and 1 mM-EGTA, favoring the activity of inositol polyphosphate 5-phosphatase (Brearley et al., 1997).

Periodate Oxidation, Reduction, and Dephosphorylation of Inositol Phosphates to Polyols

We have used the periodate oxidation, reduction, and dephosphorylation technique originally devised by Clinton Ballou (Tomlinson and Ballou, 1961) and given a contemporary reading with *myo*-[^{3}H]inositol labeled substrates (as described by Stephens [1990]) to determine the stereoisomerism of inositol phosphates identified in this study. Periodate attack on the inositol ring cleaves C—C bonds that bear cis-orientated hydroxyl groups. Subsequent reduction with borohydride yields an acyclic polyol phosphate that retains the stereochemistry of substitution of the inositol ring. Removal of phosphates with alkaline phosphatase then yields an acyclic polyol that can be resolved by HPLC and that retains the stereochemistry of substitution. Substitution of the hydroxyl functions of the parent inositol ring with phosphate can thus be seen to protect C—C bonds from periodate attack. Since within a particular class of inositol phosphates (e.g. trisphosphates, of which there are 20 possible non-cyclic stereoisomers) there are several stereoisomers that yield the same polyol, the identification of the parent(s) often relies on additional chromatographic information (i.e. the separation of parent inositol phosphates prior to oxidation).

Inositol phosphates were oxidized with sodium periodate, pH 4.0, reduced, and dephosphorylated (Stephens and Downes, 1990). After reduction, approximately 50 μg each of a mixture of unlabeled polyols: inositol, ribitol, D-arabitol, D-altritol, xylitol, D-glucitol, and L-iditol, was added to act as a carrier through subsequent processing steps. Polyols were resolved on either Polypore Pb^{2+} (Brownlee, Applied Biosystems, Santa Clara, CA) HPLC columns or NH_2 HPLC columns (Microsorb, Woburn, MA). For Polypore HPLC the column was held at 60°C, the injection volume was 30 to 50 μL, and the flow rate was 0.2 mL/min. The NH_2 column was maintained at ambient temperature. The sample was injected in 50 μL of acetonitrile:water (50:50, v/v) and the column eluted with 65% acetonitrile (v/v) in water (Brearley and Hanke, 1992). Throughout HPLC absorbance was monitored at 190 nm. Fractions were collected (see figures for details), and either aliquots or total fractions were counted for ^{3}H and ^{14}C.

Standards

[^{32}P]Ins(1,4,5) P_3 and [^{32}P]Ins(2,4,5) P_3 were prepared by alkaline hydrolysis of PtdIns(4,[^{32}P]5) P_2, the product of a purified PtdIns4P 5-kinase. Alternatively, [^{32}P]PtdIns(4,5) P_2 was prepared from [^{32}P]Pi-labeled turkey erythrocyte lipids. [^{32}P]Ins(2,4,5) P_3 and traces of [^{32}P]Ins(3,4,5) P_3 were also obtained by mild acid treatment of [^{32}P]Ins(1,4,5) P_3. [^{14}C]Ins(3,4,6) P_3 was obtained from [^{14}C]inositol-labeled *S. polyrhiza* (Brearley and Hanke, 1996a). [^{14}C]Ins(1,2,3) P_3 and D- and/or L-[^{14}C]Ins(1,2,6) P_3 were prepared by treatment of [^{14}C]InsP_6 obtained from *S. polyrhiza* with a commercial preparation (Sigma) of wheat bran phytase essentially according to the method of Stephens (1990).

Permeabilization Experiments

Mesophyll protoplasts were prepared from *C. communis* and electroporated (Brearley et al., 1997) at a cell density of approximately 2×10^7 cells/mL in 0.3 mL of medium containing 4.5×10^5 dpm of [^{3}H]Ins(3,4,5,6) P_4 obtained from *S. polyrhiza* (Brearley and Hanke, 1996a), supplemented in some cases with 20 mM MgATP. Glc 6-P was included at a 20 mM concentration to guard against the potential breakdown of added inositol phosphates by unspecified enzyme activities. Protoplasts were incubated for 7 min following electroporation and quenched with perchloric acid.

RESULTS

Multiple Isomers of InsP_3 in *S. polyrhiza*

Partisphere SAX HPLC of perchloric acid extracts from [^{3}H]inositol-labeled *S. polyrhiza* resolved three peaks of InsP_3 (Fig. 1). Peak I eluted before [^{32}P]Ins(3,4,5) P_3, [^{32}P]Ins(1,4,5) P_3, and [^{32}P]Ins(2,4,5) P_3; peak II eluted after [^{32}P]Ins(3,4,5) P_3, with [^{32}P]Ins(1,4,5) P_3, and before [^{32}P]Ins(2,4,5) P_3; while peak III eluted after both [^{32}P]Ins(3,4,5) P_3 and [^{32}P]Ins(1,4,5) P_3 and with [^{32}P]Ins(2,4,5) P_3. We have previously shown that peak III contains Ins(3,4,6) P_3 (Brearley and Hanke, 1996a).

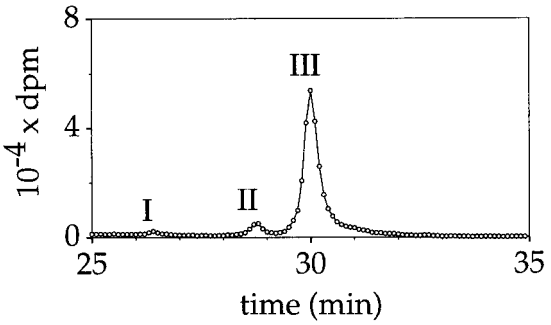

Figure 1. Inositol trisphosphates in *S. polyrhiza*. Inositol trisphosphates from [^{3}H]inositol-labeled *S. polyrhiza* were separated by HPLC and radioactivity monitored with a flow detector (Radiomatic series A-500, Canberra Packard, Pargbourne, Berks, UK). Separations such as this have been observed in more than 10 experiments.

Ins(1,2,3) P_3 Is Present in Vegetative Tissues of Monocots

The polyol products of periodate oxidation, reduction, and dephosphorylation of peak I were mixed with standards and resolved on Polypore Pb^{2+} HPLC (Fig. 2A). A single major peak of ^{3}H-labeled material was detected and this eluted precisely with [^{14}C]ribitol and before standards of arabitol, altritol, xylitol, glucitol, and iditol. However, because of the difficulty in obtaining separations of ribitol and inositol on Polypore Pb^{2+} columns, aliquots of peak fractions containing [^{14}C]ribitol and ^{3}H-label were pooled, freeze-dried, and re-run on an aminopropyl column (Brearley and Hanke, 1992). Under these conditions the ^{3}H-label co-eluted precisely with [^{14}C]ribitol and before [^{14}C]inositol. This identifies the parent inositol phosphate as Ins(1,2,3) P_3.

Ins(1,4,5) P_3, if Present, Is Only a Minor Component

The identity of the inositol phosphate(s) in peak II was pursued in several ways. It was desalted and co-presented with an authentic standard of [^{32}P]Ins(1,4,5) P_3 to a preparation of human erythrocyte ghosts, and samples of the reaction products were withdrawn at intervals for HPLC. The results (not shown) revealed that the kinetics of metabolism of the ^{3}H- and ^{32}P-labeled compounds to products with the chromatographic properties of $InsP_2$s, were very different. The principal [^{3}H]$InsP_2$ product of dephosphorylation eluted after [^{32}P]Ins(1,4) P_2. Although this analysis cannot discount the possibility that the parent ^{3}H peak contains some Ins(1,4,5) P_3, the indication is that the major component is not Ins(1,4,5) P_3. The analysis does suggest, however, that a component in the peak bears a phosphate in the five position.

Identification of Inositol Phosphates Novel to Plants: D- and/or L-Ins(3,4,5) P_3 and D- and/or L-Ins(3,5,6) P_3

Another preparation of [^{3}H]$InsP_3$ peak II was resolved on Partisphere SAX HPLC, desalted, and applied to an Adsorbosphere SAX HPLC column (Brearley and Hanke, 1996b) on which a single peak of ^{3}H label was resolved. Periodate oxidation, reduction, and dephosphorylation of this peak yielded a major product that co-eluted precisely with xylitol, but after inositol, arabitol, and altritol, and before glucitol and iditol (Fig. 2B). Smaller amounts of ^{3}H label co-eluted precisely with inositol, glucitol, and iditol. We have not determined the enantiomeric identity of the polyols obtained.

Because the parent peak co-eluted with Ins(1,4,5) P_3 but before both Ins(3,4,6) P_3 and Ins(2,4,5) P_3 on Partisphere SAX HPLC [see Stephens and Downes (1990) and Stephens and Irvine (1990) for separation of Ins(1,4,5) P_3 from Ins(3,4,6) P_3, and Chilvers et al., (1991), for separation of Ins(1,4,5) P_3 from Ins(2,4,5) P_3], the production of xylitol identifies the major component of this peak as D- and/or L-Ins(1,5,6) P_3. The glucitol-yielding component is D- and/or L-Ins(1,2,5) P_3, since D- and/or L-Ins(2,4,5) P_3, the

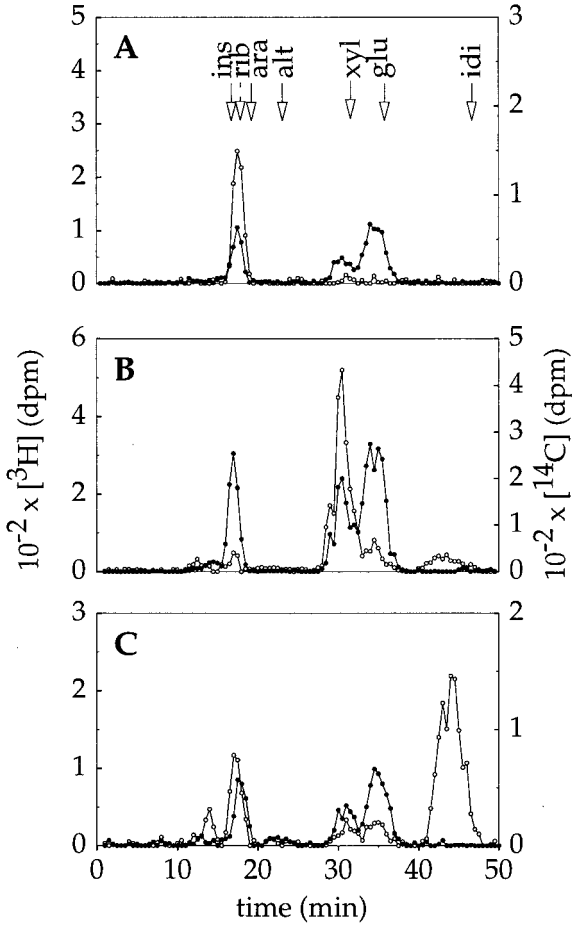

Figure 2. HPLC of polyols derived from $InsP_3$s. Polyols from [^{3}H]$InsP_3$s peaks I, II, and III (Fig. 1) were mixed, respectively, with [^{14}C]ribitol, xylitol, and glucitol (A); [^{14}C]inositol, xylitol, and glucitol (B); or [^{14}C]ribitol, xylitol, and glucitol (C), and resolved by HPLC. Fractions were collected and radioactivity in aliquots was determined. Unlabeled polyol standards, monitored at 190 nm, are indicated with arrows. ^{3}H, ○; ^{14}C, ●. ins, Inositol; rib, ribitol; ara, arabitol; alt, altritol; xyl, xylitol; glu, glucitol; idi, iditol. Repeat analysis of polyols in [^{3}H]$InsP_3$ peaks II and III yielded similar results. Confirmation of the identity of the polyol derived from [^{3}H]$InsP_3$ peak I was provided by subsequent analysis on an aminopropyl column (see text).

only other isomers that yield glucitol (Stephens, 1990), elute after Ins(1,4,5) P_3. Because iditol, the third polyolobtained from the parent $InsP_3$ peak, is obtained from both D- and/or L-Ins(1,4,5) P_3 and D- and/or L-Ins(3,4,6) P_3, the elution of $InsP_3$ II before Ins(3,4,6) P_3 (the major peak in Figure 1), leaves D- and/or L-Ins(1,4,5) P_3 as the only possible parents for the iditol obtained. That this minor peak eluted before Ins(3,4,6) P_3, which was present in excess over the others, means that the iditol obtained from the second peak could not be derived from peak III.

D- and/or L-Ins(2,4,5) P_3 Is Present in Plants

We also performed an analysis of peak III in an attempt to identify any minor components in this peak. The peak was desalted, subjected to periodate oxidation, reduction, and dephosphorylation, and the products were resolved on HPLC (Fig. 2C). Among the potential products of oxidation of authentic $InsP_3$s, four peaks of ^{3}H-polyols were detected. The first eluted just before [^{14}C]ribitol and is likely to be [^{3}H]inositol. Its presence is either the result of a failure in the oxidation of the $InsP_3$s in the parent peak (peak III) or is indicative of the presence of Ins(1,3,5) P_3 or Ins(2,4,6) P_3, which are both resistant to periodate oxidation. We are not aware of the identification of Ins(1,3,5) P_3 or Ins(2,4,6) P_3 in either plants or animals.

The second polyol, a minor component, co-eluted precisely with [^{14}C]xylitol. The third, also a minor component co-eluted precisely with D-[^{14}C]glucitol. No attempt was made to determine the enantiomerism of the [^{3}H]glucitol product, whereas xylitol and *myo*-inositol are both *meso*-compounds. The fourth and major peak co-eluted with iditol. The enantiomerism of this product was characterized previously (Brearley and Hanke, 1996a). The parent inositol phosphate is Ins(3,4,6) P_3.

The presence of a trace of material that eluted with [^{14}C]xylitol is perhaps more difficult to explain. The only *myo*-[2-^{3}H]inositol-labeled $InsP_3$s that yield [^{3}H]xylitol on oxidation are D/L-Ins(1,5,6) P_3 (Stephens, 1990). Xylitol was identified as the major product of oxidation of peak II, consequently the most likely explanation of the presence of [^{3}H]xylitol in the products of oxidation of peak III is the incomplete resolution of peaks II and III on Partisphere SAX HPLC and on subsequent fractionation, i.e. the tailing of peak II into peak III.

The presence of [^{3}H]glucitol in the products of peak III is unlikely to be explained by the tailing of peak II into peak III, since glucitol was only a minor component relative to xylitol in the products of treatment of peak II (Fig. 2B), whereas it was present in approximately equal amounts to xylitol in the products of treatment of peak III. We conclude that the [^{3}H]glucitol derived from peak III is indicative of the presence of D- and/or L-Ins(2,4,5) P_3, which elutes with D/L-Ins(1,4,6) P_3 on Partisphere SAX HPLC (14). D- and/or L-Ins(2,4,5) P_3 has been detected in avian erythrocytes (Radenberg et al., 1989), in which it is one of the major isomers in terms of chemical mass, and possibly in rat mammary tumor cells (Wong et al., 1992).

$InsP_3$s in Arabidopsis: Identification of Ins(1,2,3) P_3 and D- and/or L-Ins(3,4,6) P_3

When extracts from labeled Arabidopsis cultures were mixed with [^{14}C]Ins(1,2,3) P_3, D- and/or L-[^{14}C]Ins(1,2,6) P_3, and [^{14}C]Ins(3,4,6) P_3, three distinct peaks of [^{3}H]$InsP_3$ were resolved (Fig. 3A). The first ^{3}H peak co-eluted precisely with [^{14}C]Ins(1,2,3) P_3 and, given the diagnostic very early-eluting nature of the latter compound on Partisphere SAX HPLC, is likely to be the same. The second peak of ^{3}H label eluted after [^{14}C]Ins(1,2,3) P_3 and before [^{14}C]Ins(1,2,6) P_3, while the third co-eluted precisely with [^{14}C]Ins(3,4,6) P_3 after [^{14}C]Ins(1,2,6) P_3. In a separate HPLC run (Fig. 3B) the first peak eluted before [^{32}P]Ins(1,4,5) P_3, [^{32}P]Ins(2,4,5) P_3, and also before what we assume to be a trace of [^{32}P]Ins(3,4,5) P_3, which elutes before Ins(1,4,5) P_3 on SAX columns (Stephens and Downes, 1990). The second peak of ^{3}H label co-eluted approximately with [^{32}P]Ins(1,4,5) P_3 and before [^{32}P]Ins(2,4,5) P_3, while the third ^{3}H peak eluted after [^{32}P]Ins(2,4,5) P_3.

At this level of analysis the indications are that root cell suspension cultures of Arabidopsis contain $InsP_3$s with

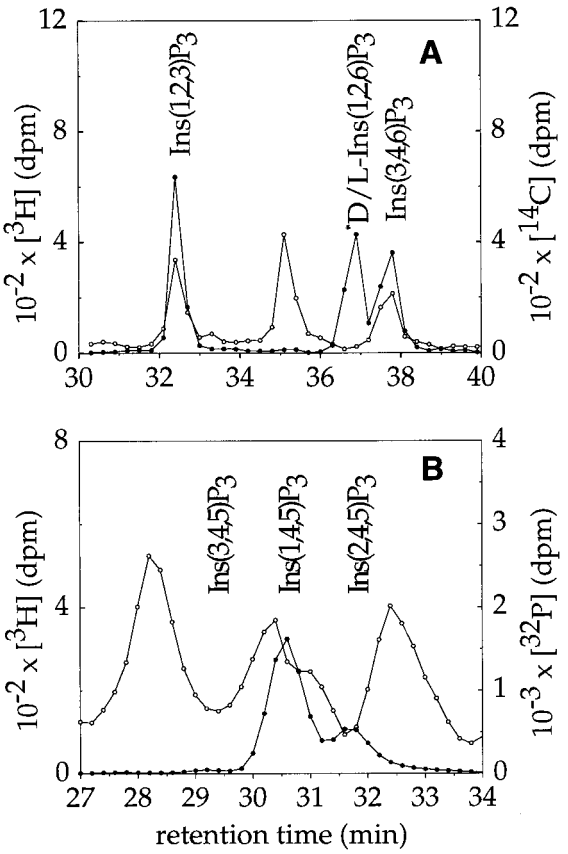

Figure 3. Inositol trisphosphates in Arabidopsis root suspension cultures. Extracts from [^{3}H]inositol-labeled Arabidopsis were mixed with standards and resolved by HPLC. Radioactivity in column fractions was determined by dual-label scintillation counting (A: ^{3}H, ○; ^{14}C, ●), and by on-line scintillation counting (B: ^{3}H, ○; ^{32}P, ●). The data in B were five-point-smoothed by the flow-detector software. The peaks identified are the internal standards. Separation of three $InsP_3$ peaks was confirmed in an independent experiment.

the chromatographic properties of Ins(1,2,3) P_3 and Ins(3,4,6) P_3. The other peak was not identified, but we can exclude Ins(1,2,3) P_3, D/L-Ins(1,5,6) P_3, D/L-Ins(2,4,5) P_3, D/L-Ins(1,2,6) P_3, D/L-Ins(1,4,6) P_3, and probably also D/L-Ins(1,3,4) P_3, which elutes before D/L-Ins(1,5,6) P_3 on SAX columns (Stephens and Downes, 1990), as potential identities.

Ins(3,4,5,6) P_4 6-Phosphatase and Ins(3,4,5,6) P_3 4-Phosphatase Activities in Mesophyll Protoplasts

To determine the origins of D- and/or L-Ins(1,5,6) P_3 and D- and/or L-Ins(1,4,5) P_3 components of peak II, and bearing in mind the identification of Ins(3,4,5,6) P_4 in *S. polyrhiza* (Brearley and Hanke, 1996a), we considered the possibility that D- and/or L-Ins(1,5,6) P_3 [L-Ins(1,5,6) P_3 = D-Ins(3,4,5) P_3] and D- and/or L-Ins(1,4,5) P_3 [L-Ins(1,4,5) P_3 = D-Ins(3,5,6) P_3] might be the product(s) of dephosphorylation of Ins(3,4,5,6) P_3. We permeabilized preparations of mesophyll protoplasts in the presence of [^{3}H]Ins(3,4,5,6) P_4. We rationalized the choice of experimental material in that the inositol phosphates identified in *S. polyrhiza* are probably those of the frond mesophyll tissue, which represents the major part of this much reduced and highly specialized plant. We prepared mesophyll protoplasts from the leaves of the monocot *C. communis* from which, unlike *S. polyrhiza*, the abaxial epidermis is easily stripped, easing the preparation of protoplasts.

Mesophyll protoplasts permeabilized in the presence of [^{3}H]Ins(3,4,5,6) P_4 gave two small peaks of $InsP_3$ (Fig. 4A). Supplementation of the basic electroporation medium (ATP concentration, 60 μM) with 20 mM MgATP stimulated production of an $InsP_5$. The generation of $InsP_3$s in other experiments in which we did not detect phosphorylation of $InsP_4$ suggests that the $InsP_3$s detected are the products of direct dephosphorylation of Ins(3,4,5,6) P_4. Of the potential products of direct dephosphorylation of Ins(3,4,5,6) P_4, three [Ins(3,4,5) P_3, Ins(3,5,6) P_3, and Ins(3,4,6) P_3] can be resolved in order of increasing retention time on Partisphere SAX HPLC (Stephens and Downes, 1990; Stephens and Irvine, 1990; Brearley and Hanke, 1996a, 1996b). Ins(4,5,6) P_3 also elutes after Ins(1,4,5) P_3, the enantiomer of Ins(3,5,6) P_3 (Stephens and Downes, 1990). By inclusion on Partisphere SAX HPLC of a standard of [^{14}C]Ins(3,4,6) P_3, we found (Fig. 4B) that the two $InsP_3$ products of metabolism of [^{3}H]Ins(3,4,5,6) P_4 eluted before [^{14}C]Ins(3,4,6) P_3, which eluted before a trace of late-eluting material. The HPLC run also included ^{14}C-labeled standards of Ins(1,2,3) P_3 and D- and/or L-Ins(1,2,6) P_3. According to the known chromatographic properties of $InsP_3$s on SAX columns, we identified the first two $InsP_3$s as Ins(3,4,5) P_3 and Ins(3,5,6) P_3, in order of increasing elution time, and the third as Ins(4,5,6) P_3. The identification of the same $InsP_3$s, albeit as stereoisomers and not individual enantiomers, in *S. polyrhiza* provides strong evidence that D- and/or L-Ins(1,5,6) P_3 and D- and/or L-Ins(1,4,5) P_3 found therein are products of metabolism of Ins(3,4,5,6) P_4. The foregoing also explains the origins of D- and/or L-Ins(1,4,5) P_3 in *S. polyrhiza* and suggests that the isomer might be the L-enantiomer [D-Ins(3,5,6) P_3].

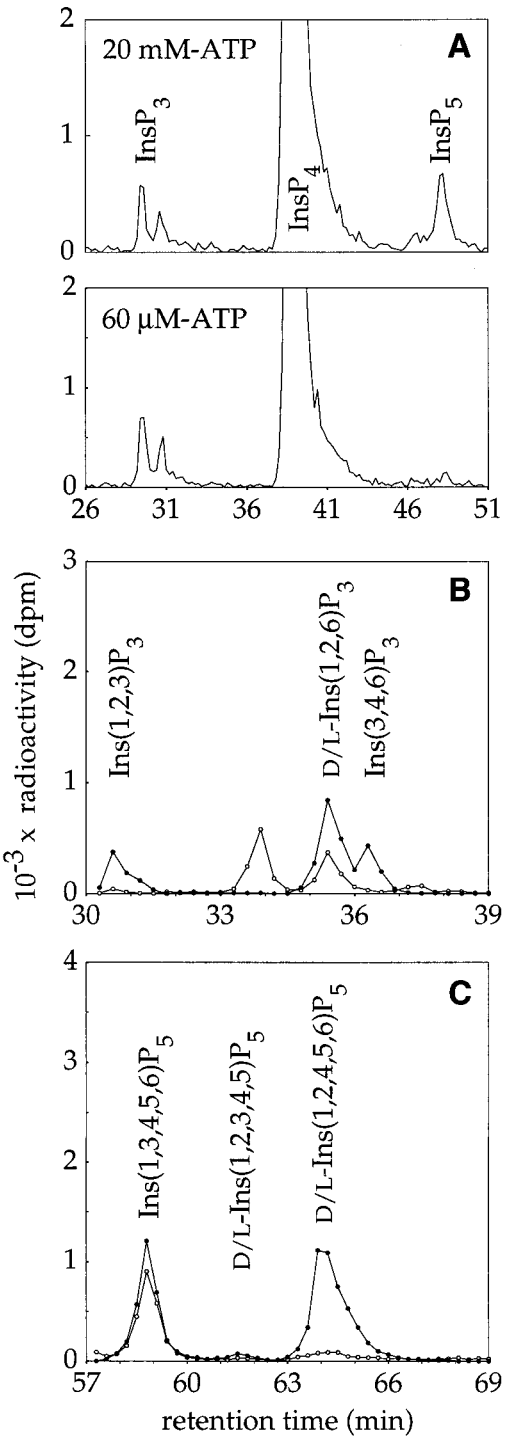

Figure 4. Metabolism of Ins(3,4,5,6) P_4 by permeabilized mesophyll protoplasts. Extracts from protoplasts permeabilized with [^{3}H]Ins(3,4,5,6) P_4, with or without supplemental ATP, were resolved by HPLC (A). The traces are normalized with respect to the $InsP_4$ peak. HPLC traces of extracts mixed with [^{14}C]$InsP_3$ and $InsP_5$ standards are shown in B and C. ^{3}H, ○; ^{14}C, ●. The peaks identified are the internal standards. Phosphorylation of Ins(3,4,5,6) P_4 to $InsP_5$ was repeated in two additional experiments. Two peaks of $InsP_3$ were generated in all four experiments.

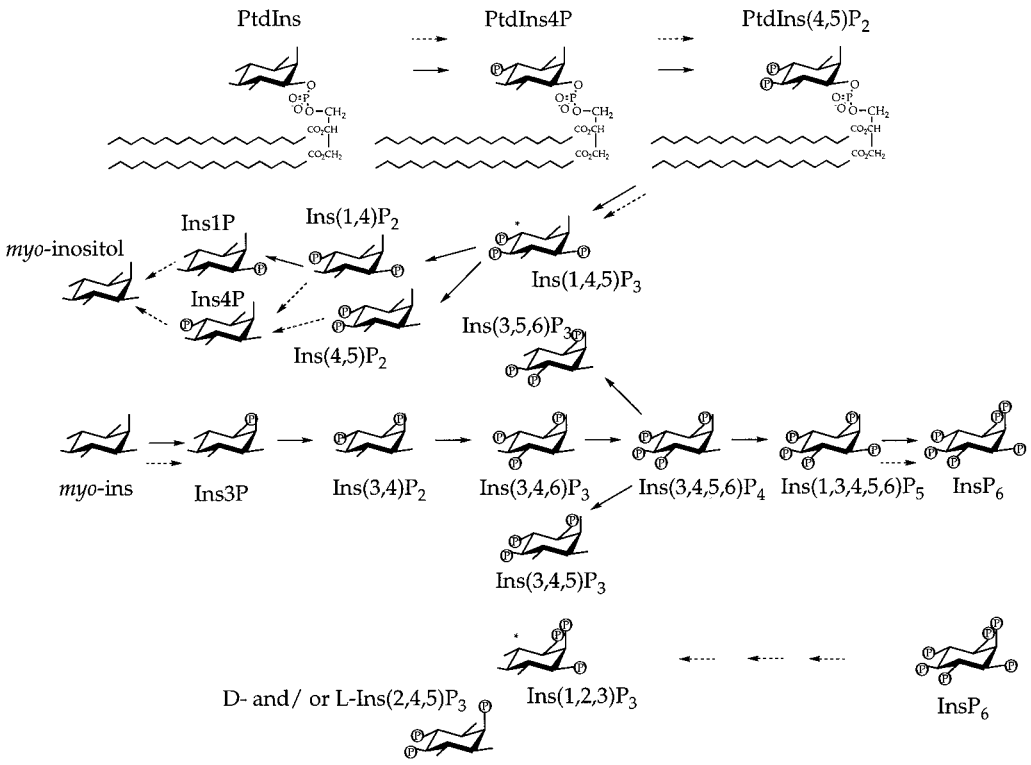

Figure 5. Inositol phosphates and a scheme for their metabolism in mesophyll cells. Steps for which there is direct metabolic evidence are indicated with solid arrows. Reactions that have been demonstrated in other experimental systems in vitro are indicated with dashed arrows. Asterisks (*), $InsP_3$s common to monocots and dicots.

Ins(3,4,5,6) P_4 1-Kinase Activity in Mesophyll Protoplasts

The $InsP_5$ product of metabolism of [^{3}H]Ins(3,4,5,6) P_4 was chromatographed (Fig. 4C) against internal standards of [^{14}C]$InsP_5$, as described previously (Brearley and Hanke, 1996a, 1996b). The order of elution of $InsP_5$s on Partisphere SAX columns is Ins(1,3,4,5,6) P_5 < D/L-Ins(1,2,3,4,5) P_5 < Ins(1, 2, 3, 4, 6) P_5 < D/L-Ins(1,2,4,5,6) P_5 (Stephens et al., 1991). Thus, the precise co-elution of the [^{3}H]$InsP_5$ product with an internal standard of [^{14}C]Ins(1,3,4,5,6) P_5 and before standards of D/L-[^{14}C]Ins(1,2,3,4,5) P_5 and D/L-[^{14}C]Ins(1,2,4,5,6) P_5 identifies the product as Ins(1,3,4,5,6) P_5 and so defines Ins(3,4,5,6) P_4 1-kinase activity in mesophyll protoplasts. This provides a convincing explanation of the metabolic origin of Ins(1,3,4,5,6) P_5 in plants and *S. polyrhiza* in particular.

DISCUSSION

Inositol Trisphosphates in Plants

Detailed analysis of inositol trisphosphates in two plants has revealed a complex array of isomers. None of the isomers detected is unique to plants though D/L-Ins(2,4,5) P_3 and Ins(1,2,3) P_3 have only occasionally been reported in vivo in any kingdom (Brearley and Hanke, 1992, 1996b; Barker et al., 1995). Although Ins(1,4,5) P_3 is one of only a few $InsP_3$s identified to date, it appears that this isomer is only a minor component of the inositol phosphate complement of higher plants.

A schematic diagram of the metabolic relationships of $InsP_3$ species identified in mesophyll cells is indicated in Figure 5. In this context, it is worth remembering that the storage tissue of the turion of *S. polyrhiza* is a specialized form of the mesophyll of the frond.

Metabolic Origins of Inositol Trisphosphates in Plants: $InsP_3$s as Products of Catabolism

The existing literature provides clues to the origins of $InsP_3$s in plants. The work of Johnson and Tate (1969), Lim and Tate (1971, 1973), and Tomlinson and Ballou (1961, 1962) reviewed by Cosgrove (1980), suggests that both Ins(1,2,3) P_3 and Ins(1,2,6) P_3 are products of $InsP_6$ breakdown in vitro. The identification of these two isomers in aleurone tissue and of Ins(1,2,3) P_3 in *S. polyrhiza* and Arabidopsis in the present study suggests that these isomers are products of $InsP_6$ metabolism (cytosolic or not) in vivo.

If D- and/or L-Ins(1,5,6) P_3 in *S. polyrhiza* turns out to be the L-enantiomeric form [D-Ins(3,4,5) P_3], then an explanation of the origin of this compound is provided by our demonstration of metabolism of Ins(3,4,5,6) P_4 to Ins(3,4,5) P_3 in mesophyll protoplasts. Other investigators (Radenberg et al., 1989) have speculated that the D- and/or L-Ins(1,5,6) P_3, which they identified in avian erythrocytes, may be a product(s) of dephosphorylation of Ins(3,4,5,6) P_4 and Ins(1,3,4,5) P_4. Our approach affords an experimental

test of this. Whereas Ins(3,4,5,6) P_4 is present in higher plants, there is no evidence yet for Ins(1,3,4,5) P_4.

Finally, in consideration of the range of $InsP_3$ isomers in plants, it is apparent that Ins(1,2,3) P_3 is common to monocots (*S. polyrhiza* and barley) and dicots (Arabidopsis). It occurs in non-highly differentiated cells (mesophyll cells) in the fronds and turions of *S. polyrhiza*, in root cortex cells of Arabidopsis, and in terminally differentiated cells in barley aleurone. It is likely that the route of Ins(1,2,3) P_3 generation is shared in monocots and dicots and perhaps also in animal cells (Barker et al., 1995), where it has been shown that Ins(1,2,3) P_3 is a product of $InsP_6$ breakdown by cell homogenates. Clearly, Ins(1,2,3) P_3 is not restricted to specialized cells or storage tissues in plants, so we should consider a general "housekeeping" function for this isomer. One such suggestion is that Ins(1,2,3) P_3 is an inhibitor of hydroxyl free radical formation (Barker et al., 1995; Phillippy and Graf, 1997).

$InsP_3$s as Intermediates in Synthetic Pathways

Ins(3,4,6) P_3 is one of the few $InsP_3$s for which there is direct evidence in vivo of the identity of its metabolic neighbors. In avian erythrocytes, Ins(3,4,6) P_3 is the precursor of Ins(3,4,5,6) P_4, which in turn is the precursor of Ins(1,3,4,5,6) P_5 (Stephens and Downes, 1990). A caveat is necessitated by the possibility, however hypothetical, of substrate cycles involving these inositol phosphates, which could compromise the interpretation of non-equilibrium labeling studies. The operation of substrate cycles for these specific isomers has not been tested directly or indirectly. Ins(3,4,6) P_3 is also an intermediate in a pathway of $InsP_6$ biosynthesis in *D. discoideum* (Stephens and Irvine, 1990).

In plants (Brearley and Hanke, 1996b), Ins(3,4,6) P_3 is an intermediate in a biosynthetic sequence to $InsP_6$, which shares the partial sequence Ins(3,4,6) $P_3 \rightarrow$ Ins(3,4,5,6) $P_4 \rightarrow$ Ins(1,3,4,5,6) P_5, which has been described in avian erythrocytes (Stephens and Downes, 1990) and may represent steps in a route of synthesis of $InsP_6$ in the animal kingdom. An alternative possibility (Shears, 1996) places Ins(1,3,4) P_3 at a branch point in inositol phosphate metabolism leading either to inositol through the action of inositol phosphate phosphatases or to $InsP_6$ via Ins(1,3,4,6) P_4 and Ins(1,3,4,5,6) P_5. Thus, the recent cloning of human (Wilson and Majerus, 1996) and Arabidopsis (Wilson and Majerus, 1997) genes whose products, tested against a limited number of substrates, show Ins(1,3,4) P_3 5/6-kinase activity in vitro is particularly interesting.

Given the lack of consensus on the route(s) of $InsP_6$ synthesis in animals, plants and *D. discoideum*, our demonstration that Ins(3,4,5,6) P_4 is a substrate for Ins(3,4,5,6) P_4 1-kinase activity in mesophyll protoplasts of monocots is entirely consistent with the earlier proposal that in plants the 1-P of $InsP_6$ is added after the 4- and 5-Ps (Brearley and Hanke, 1996b). Because the 3-P and not the 1-P is added first, such observations discount the possibility that Ins(1,4,5) P_3 or Ins(1,3,4) P_3 are precursors of $InsP_6$ in *S. polyrhiza*. We found no evidence for the presence of Ins(1,4,5) P_3-kinase activity in mesophyll protoplasts (Brearley et al., 1997) under similar experimental conditions to those described here.

Moreover, an inositol polyphosphate 5/6-kinase has been cloned from Arabidopsis (H.W. Xue, C.A. Brearley, and B. Mueller-Roeber, unpublished data) that is identical to that previously reported in Arabidopsis (Wilson and Majerus, 1997). The product of our gene shows Ins(3,4,6) P_3 5-kinase activity, consistent with the precursor-product relationship of Ins(3,4,6) P_3 and Ins(3,4,5,6) P_4 in a pathway to $InsP_6$ (Brearley and Hanke, 1996a, 1996b), and which, considering the identification of D- and/or L-Ins(3,4,6) P_3 in Arabidopsis, might suggest a common pathway in monocots and dicots. It is, however, quite possible that there are multiple routes to $InsP_6$ in plants. Phillippy (1998) has recently identified separate inositol 1,3,4-trisphosphate 5-kinase and inositol 1,3,4,5-tetrakisphosphate 6-kinase activities in immature soybean plants.

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