Inositol phosphates in the duckweed *Spirodela polyrrhiza* L.

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We have undertaken an analysis of the inositol phosphates of *Spirodela polyrrhiza* at a developmental stage when massive accumulation of $\text{Ins}P_6$ indicates that a large net synthesis is occurring. We have identified $\text{Ins}3P$, $\text{Ins}(1,4)P_2$, $\text{Ins}(3,4)P_2$ and possibly $\text{Ins}(4,6)P_2$, $\text{Ins}(3,4,6)P_3$, $\text{Ins}(3,4,5,6)P_4$, $\text{Ins}(1,3,4,5,6)P_5$, D- and/or L- $\text{Ins}(1,2,4,5,6)P_5$ and $\text{Ins}P_6$ and revealed the likely

presence of a second $\text{Ins}P_3$ with chromatographic properties similar to $\text{Ins}(1,4,5)P_3$. The higher inositol phosphates identified show no obvious direct link to pathways of metabolism of second messengers purported to operate in higher plants, nor do they resemble the immediate products of plant phytase action on $\text{Ins}P_6$.

INTRODUCTION

Until the elucidation of the pathway of *myo*-inositol hexakisphosphate (phytate; $\text{Ins}P_6$) synthesis in *Dictyostelium* [1] very little was known of the pathway of $\text{Ins}P_6$ synthesis in either the plant or animal kingdoms. By corollary the metabolic relationships with other aspects of inositol phosphate metabolism, e.g. second messenger function, were also undefined. It is now apparent that in *Dictyostelium* $\text{Ins}P_6$ synthesis from *myo*-inositol proceeds via $\text{Ins}3P$, $\text{Ins}(3,6)P_2$, $\text{Ins}(3,4,6)P_3$, $\text{Ins}(1,3,4,6)P_4$ and $\text{Ins}(1,3,4,5,6)P_5$ and that the pathway has no direct relationship with the metabolism of the second messenger $\text{Ins}(1,4,5)P_3$.

Early studies of $\text{Ins}P_6$ synthesis in plants [2,3] led to the proposal that during mung bean seed development and germination $\text{Ins}P_6$ synthesis from $\text{Ins}3P$ was mediated by phosphoinositol kinase(s) via a series of undefined inositol phosphate isomers. Subsequent work identified a phosphotransferase activity capable of adding a phosphate to the 2-position of $\text{Ins}(1,3,4,5,6)P_5$ from ATP, but the substrate specificity of the activity was not characterized [4].

Although various workers have detected a range of inositol phosphates of differing degrees of phosphorylation in a variety of experimental systems such as mung bean [2,3,5], rice cell culture [6,7], soybean [8] and duckweed [9,10], practically nothing is known of the stereochemical identities of these compounds. The situation is further complicated by the likelihood that some of the inositol phosphates detected are in fact products of $\text{Ins}P_6$ breakdown.

Another unanswered question that relates to the pathway of $\text{Ins}P_6$ synthesis in plants concerns the source of $\text{Ins}3P$. Two enzyme activities that are capable of synthesizing $\text{Ins}3P$ have been identified in plants. These are *myo*-inositol phosphate synthase (EC 5.5.1.4), which converts glucose 6-phosphate to $\text{Ins}3P$ and is the ultimate source of *myo*-inositol in plants, and *myo*-inositol kinase (EC 2.7.1.64), which converts *myo*-inositol to $\text{Ins}3P$ [11]. The spatial and temporal distribution and the relative contributions of these two enzymes to $\text{Ins}P_6$ synthesis via $\text{Ins}3P$ are unclear, although in germinating mung bean the kinase does not seem to be expressed at the same time as the enzymes catalysing $\text{Ins}P_6$ synthesis [4].

We have set out in this and the following paper [12] to define the stereochemical identities of the inositol phosphate intermediates by which $\text{Ins}P_6$ is synthesized from *myo*-inositol in the duckweed *Spirodela polyrrhiza* L.

MATERIALS AND METHODS

Reagents

myo-[2- ^3H]Inositol and [^{32}P]orthophosphate (carrier-free) were obtained from Amersham International (Amersham, Bucks., U.K.). Alkaline phosphatase (bovine intestinal, type 5521) was obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.).

Tissue

The aquatic monocotyledonous plant *Spirodela polyrrhiza* L. was maintained in axenic culture according to [13]. For labelling experiments the plant growth regulator abscisic acid (ABA) was included in the culture medium at a concentration of 0.3 μM . This treatment induces the plants to switch from vegetative growth (frond production) to the production of $\text{Ins}P_6$ -rich turions.

Radiolabelling

Labelling of *Spirodela* plants from [^3H]inositol or [^{32}P]orthophosphate was performed essentially as described [14]. Plants were labelled with *myo*-inositol for periods up to 18 days (d).

Tissue extraction

After labelling, the washed plants (typically 20–100 mg tissue) were cooled in liquid nitrogen and ground in a liquid nitrogen-cooled mortar. At this stage an aliquot (50 μl) of phytate hydrolysate (25 μg of phosphorus, prepared by the method of [15]) was added to minimize losses of inositol phosphates due to non-specific binding during extraction. The ground tissue was extracted with 0.7 ml of 3.5% (w/v) perchloric acid. The cell debris was pelleted by centrifugation for 5 min in a refrigerated Microfuge and the supernatant neutralized to pH 6–7 with 2 M KOH/50 mM Mes/10 mM EDTA. After 15 min on ice the

Abbreviation used: ABA, abscisic acid; nomenclature: we have used the nomenclature of [19] to define the enantiomerism of inositol phosphates described in this text; that is, where the enantiomers are defined the D-nomenclature is used; where inositol phosphates are obtained from uncharacterized biological sources they are referred to as D- and/or L-isomers and where a racemic mixture of enantiomers is obtained e.g. by acid hydrolysis, the inositol phosphates are referred to as D/L-isomers; d, days.

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KClO₄ precipitate was pelleted by centrifugation in a refrigerated microfuge and the supernatant filtered (0.4 µm; Alltech, Carnforth, Lancs., U.K.) before HPLC.

Charcoal treatment

In some experiments perchloric acid extracts of [³²P]P_i-labelled tissue were treated with charcoal (Sigma) to decrease the level of contaminating nucleotides. The charcoal was washed thoroughly with ice-cold 5% perchloric acid and employed exactly as described [16].

Acid-catalysed phosphate migration of inositol phosphates

Migration of phosphate across the *cis*-related 1- and 2- and the 2- and 3-hydroxyl groups of inositol phosphates was achieved by treatment of desalted preparations of inositol phosphates with 1.0 M HCl by boiling for 10 min [17]. Reactions were quenched by freezing and HCl was removed by freeze-drying.

HPLC of neutralized perchloric acid extracts

Neutralized extracts were applied (1 ml loop) to a 25 cm Partisphere SAX HPLC column and AX guard cartridge (Whatman) by using the gradient described in [1]. The column was eluted with a gradient derived from buffers A (water) and B (2.5 M NaH₂PO₄) at a flow rate of 1 ml/min: 0 min, 0% B; 60 min, 100% B; 70 min, 100% B. In some experiments after the injection of sample the column was washed with water for 5 min before starting the gradient.

Isomer separations

InsP species were separated on Partisphere SAX eluted isocratically with 40 mM NaH₂PO₄ by the method of [18].

InsP₂ separations were performed on Partisphere SAX eluted isocratically with 240 mM NaH₂PO₄ by the method of [18].

InsP₃ separations were commonly performed on Partisphere SAX eluted isocratically with 550 mM NaH₂PO₄ by the method of [18] or on a weak anion-exchange column (10 cm Partisphere WAX, Whatman) eluted with the following gradient: A (water)/B [0.5 M (NH₄)₂HPO₄, pH 3.2 with H₃PO₄] at a flow rate of 1 ml/min: 0 min, 0% B; 10 min, 10% B; 60 min, 15% B; 70 min, 15% B; 80 min, 50% B.

InsP₄ separations were routinely performed on Partisphere SAX eluted with the following gradient: A (water)/B (2 M NaH₂PO₄, pH 4.4 with NaOH) at a flow rate of 1 ml/min: 0 min, 0% B; 1 min, 35% B; 85 min, 50% B; 86 min, 100% B; 91 min, 100% B.

InsP₅ species were separated by the method of [16] or [19] on Partisphere SAX with either of the following gradients: A (water)/B (2.5 M NaH₂PO₄, pH 3.75 with NaOH) at a flow rate of 1 ml/min: 0 min, 0% B; 1 min, 40% B; 60 min, 70% B; 61 min, 100% B; 70 min, 100% B; or A (water)/B [1.25 M (NH₄)₂HPO₄, pH 3.8 with H₃PO₄] at a flow rate of 1 ml/min: 0 min, 0% B; 10 min, 48% B; 60 min, 80% B.

Desalting of HPLC fractions

Fractions containing phosphate were diluted 5–10-fold with water adjusted to pH 6–7 with triethylamine and desalted by the method of [20].

Ghost preparation

Human erythrocyte ghosts were prepared by the method of [21] and stored at –70 °C until used. Assays using erythrocyte ghosts

were performed according to [14], that is, either in the presence of 12.5 mM Hepes, pH 7.0, containing 1 mM EGTA and 10 mM MgCl₂, or in a buffer comprising 12.5 mM Hepes, pH 7.0, 1 mM EGTA, 5 mM EDTA.

Standards

InsP species

D/L-[³H]Ins1P or [³²P]Ins1P and [³²P]Ins2P were prepared from [³H]PtdIns or [³²P]PtdIns (obtained from *Spirodela*) by acid hydrolysis by the method of [22]. Alternatively, D/L-[³H]Ins1P or [¹⁴C]Ins1P and [¹⁴C]Ins2P were prepared in a similar manner from D-[2-³H]Ins1P and D-[¹⁴C]Ins3P, obtained from Amersham International.

InsP₂ species

[³H]Ins(1,4)P₂ was obtained by erythrocyte ghost treatment of [³H]Ins(1,4,5)P₃ in the presence of MgCl₂ and the absence of EDTA. The products were purified by SAX HPLC. [³H]Ins(4,5)P₂ was prepared by limited alkaline phosphatase treatment of [³H]Ins(1,4,5)P₃ and HPLC purification of the products.

InsP₃ species

[³H]Ins(1,4,5)P₃ was obtained from New England Nuclear–DuPont (Stevenage, Herts., U.K.). [³²P]Ins(1,4,5)P₃ was generously given by Dr. L. R. Stephens (Biochemistry Department, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, U.K.) or was obtained from [³²P]P_i-labelled turkey erythrocytes after deacylation of a phospholipid extract and subsequent deglyceration of glycerophosphoinositol 4,5-bisphosphate.

InsP₄ species

[³H]Ins(1,3,4,5)P₄ was obtained from New England Nuclear–DuPont. [³²P]Ins(1,3,4,5)P₄, [³²P]Ins(3,4,5,6)P₄ and [³²P]Ins(1,3,4,6)P₄ were generously given by Dr. L. R. Stephens.

InsP₅ species

D- and/or L-[³²P]Ins(1,2,3,4,5)P₅ and D- and L-[³²P]Ins(1,2,4,5,6)P₅ were obtained from [³²P]orthophosphate-labelled mung bean seedlings labelled for 60 h, extracted and purified [16].

InsP₆

[³²P]InsP₆ was obtained from [³²P]orthophosphate-labelled mung bean seedlings.

Polyols

myo-Inositol and erythritol were purchased from Sigma. D-Iditol and ribitol were prepared from D-idose and D-ribose (purchased from Sigma) by reduction with sodium borohydride [23]. Similarly, [¹⁴C]ribitol was prepared from D-[1-¹⁴C]ribose (Amersham). D-[¹⁴C]inositol and D-[¹⁴C]glucitol were generously given by Dr. L. R. Stephens.

Production of polyols from [³H]inositol phosphates

Polyols were obtained from [³H]inositol phosphates by periodate oxidation of the inositol phosphates, reduction and dephosphorylation [23].

Periodate oxidation of inositol phosphates

Freeze-dried and desalted [^3H]inositol phosphates were incubated in 0.5 ml of 0.1 M periodic acid (adjusted to pH 2 with NaOH) for 36 h at 25 °C in a closed vessel in the dark. The aldehydes produced by oxidation and the excess periodic acid were reduced by the addition of 0.5 ml of 1.0 M sodium borohydride and further incubation for 12 h at 25 °C in an open vessel. Unlabelled polyols (usually 50 μg each of *myo*-inositol, *meso*-threitol, D-arabitol, D-altritol, D-xylitol, D-glucitol and L-iditol) were added and the sample was passed through a 3 ml column of Dowex AG-50W X4; 200–400 mesh resin, H^+ form (Sigma). After the sample had been freeze-dried, the boric acid produced on acidification was taken up in 10 ml of methanol and removed as trimethylborane by freeze-drying. Traces of boric acid remaining were removed by a second cycle of methylation and freeze-drying.

Dephosphorylation of products of periodate oxidation and reduction

The products of vigorous periodate oxidation and reduction of [^3H]inositol phosphates were dephosphorylated by incubation with alkaline phosphatase. Samples were incubated with alkaline phosphatase (10 units/ml, Type 5521, units defined by Sigma) in 10 mM ethanolamine/HCl, pH 9.5, containing 1 mM MgSO_4 for 12 h at 25 °C. The [^3H]polyols produced were desalted on a 2 ml column of Amberlite MB-3 resin. The samples were left to stand on the column for 45 min before the column was drained and washed with 5 ml of water. The column eluates were combined and freeze-dried.

HPLC of polyols

Resolution of polyols was achieved on a cation-exchange column, Polypore Pb^{2+} (22 cm $\times$ 0.46 cm with a 3 cm guard cartridge of the same material), purchased from Owens Polyscience Ltd. (Macclesfield, Cheshire, U.K.). The mobile phase was deionized water run at ambient temperature at a flow rate of either 0.2 or 0.4 ml/min. The sample loop volume was either 20 or 50 μl . Detection of 50 μg of unlabelled polyol standards was achieved in a run parallel to that used to separate radiolabelled polyols by monitoring the UV absorbance at 200 nm on a Spectra Physics 200 UV detector. Radiolabelled polyols were detected in fractions by measuring the radioactivity after the addition of 4 ml of Hionic Fluor scintillation fluid (Canberra Packard Ltd.). With the exception of erythritol and ribitol (adonitol), it is possible to separate on Polypore Pb^{2+} all the polyols that would be derived from all of the possible non-cyclic *myo*-inositol phosphates [17]. We were unable to resolve completely standards of inositol and ribitol, or ribitol and erythritol (see also [24], but see [25] for separation of these compounds on an NH_2 -column).

Use of polyol dehydrogenase to determine D-/L-isomerism of [^3H]polyols obtained from inositol phosphates

To establish the stereochemical identity of some of the inositol phosphates it was necessary to determine the D-/L-isomerism of the polyols produced by the above procedures. As a given D- or L-polyol may be obtained from more than one InsP_4 , for example, the derivation of a particular polyol does not necessarily provide an unambiguous identification of the parent inositol phosphate. However, by the exclusion of other potential inositol phosphate parent compounds on chromatographic grounds, it is possible to arrive at an unambiguous answer. The D-/L-isomerism of the polyol products of oxidation, reduction and dephosphorylation

of some of the [^3H]inositol phosphates was determined by use [23] of a commercially available L-iditol dehydrogenase: L-iditol:NAD $^+$ oxidoreductase; EC 1.1.1.14 (Sigma). Polyol-containing fractions from Polypore Pb^{2+} columns were freeze-dried. The polyols were presented as substrate to L-iditol dehydrogenase (2 units/ml) in an assay (1 ml volume) containing 100 mM Tris/HCl (pH 8.3, 22 °C), 20 mM β -NAD $^+$ and L-iditol at a final concentration of 200 μM . The reaction was followed by monitoring A_{340} . After 2–3 h, when approx. 40–80 % (as determined subsequently) of the starting L-iditol had been metabolized, the assay mixture was transferred to a Microfuge tube and boiled for 3 min to stop the reaction. The sample was centrifuged for 5 min at full speed in a Microfuge and the supernatant desalted on Amberlite MB-3 resin as described above. The desalted and freeze-dried products of the iditol-dehydrogenase assay were reapplied to a Polypore Pb^{2+} HPLC column.

The extent of metabolism of the [^3H]polyol was determined from the distribution of radioactivity between the iditol substrate and the sorbose product. The extent of metabolism of unlabelled L-iditol substrate was estimated by fitting the progress of reaction (A_{340}) curve to an equation describing a first-order reaction, which, by assuming a 1:1 stoichiometry of L-iditol oxidation to NAD $^+$ reduction, allowed extrapolation of the reaction curve to completion and hence determination of the extent of the reaction at the time it was stopped.

RESULTS

Physiology of turion induction and phytic acid accumulation in *Spirodela*

Spirodela polyrhiza, a floating duckweed, is a member of the Lemnaceae which under appropriate environmental conditions, such as low nitrate, high CO_2 , or short daylength, forms perennating structures or turions [26]. These overwintering structures, which are the functional and morphological equivalent of the dormant tubers of some land plants, abscise from the parent frond and sink to the substratum. In spring, under appropriate environmental conditions, the turions germinate and the resulting plants reproduce vegetatively. The ultrastructure of the turion has been extensively studied [13,27]. Its morphology, although typical of the frond tissue with which it shares a common progenitor in the frond primordium, is characterized by heavily thickened cell walls and the accumulation of starch grains and phytin granules.

In the laboratory the developmental switch between frond and turion production can be triggered by administering the plant hormone ABA [13,26–28]. Within 3 d of treatment the developing turions are evident and by 7 d most have abscised. We have found that to induce turion formation reliably in cultures of *Spirodela* plants the culture must be at least 3–4 weeks old. It is not clear whether this change in sensitivity of the target tissue, the frond primordia, is a consequence of developmental effects with their origin in nutrient limitation in the culture, the generation of a conditioning factor by the plants themselves, or some other parameter. Nevertheless the characteristic morphology of the turion gives an unmistakable indication of the success of turion induction by ABA. The data presented below show the pattern of inositol-labelled peaks that can be detected on Partisphere SAX HPLC of extracts prepared from *myo*-[2- ^3H]inositol-labelled *Spirodela* cultures.

HPLC of perchloric acid extracts from *Spirodela*

HPLC of perchloric acid extracts of frond tissue from control plants typically yielded a chromatographic profile that included

Figure 1 Induction of InsP_6 synthesis by ABA in *Spirodela polyrhiza*

Spirodela polyrhiza plants were labelled from $\text{myo-}[^3\text{H}]\text{inositol}$ in the presence or absence of $0.3 \mu\text{M}$ ABA. At intervals perchloric acid extracts were prepared and aliquots of these applied to a Partisphere SAX column eluted with a gradient of NaH_2PO_4 as described in the Materials and methods section. The ^3H content of the column eluate was determined by online scintillation counting with a Canberra Packard Radiomatic A500 series flow detector fitted with a 2 ml flow cell, after mixing the HPLC eluate with Canberra Packard Flo Scint IV scintillation cocktail. Scintillation events were counted with an integration interval of 6 s. Peaks in regions of the chromatogram corresponding to the approximate positions of elution of inositol phosphates bearing 1 to 6 phosphates are shown. Identical patterns of inositol phosphates were obtained from control and ABA-treated tissues in over ten separate experiments. (a) 6 d control frond tissue; (b) 6 d ABA frond tissue; (c) 6 d ABA turion tissue; (d) 18 d ABA turion tissue.

all the peaks detailed in Figure 1. That is, a number of peaks of labelled material of increasing retention time and presumably increasing phosphorylation were observed. The same peaks were present in frond tissue labelled for periods of 1–18 d. These peaks were tentatively identified by reference to the data of [1]. The rigorous determination of the identity of these peaks is described below. Analysis of equivalent material from ABA-treated tissue is also shown in Figure 1. At labelling periods of 4 d or less the frond and developing turion tissue were extracted together. At 6 d or longer the almost completely formed turions were dissected

from the parent fronds and extracted with those that had abscised. A comparison of the pattern of inositol-labelled peaks obtained in control frond and ABA-treated turion tissue reveals a dramatic switch (note the change in y-axis scale) in metabolism such that at 6 d a massive accumulation of InsP_6 is evident and this is considerably increased by 18 d, when InsP_6 accounts for over 80 % of the ^3H inositol label in the extract. Although ABA treatment caused dramatic changes in the relative sizes of the peaks detected, it did not generate any peaks that were not present in control tissue. The considerable resolving power of this Partisphere SAX gradient, particularly for higher phosphates of inositol, is evident in the data of [1] whose gradient we used. In some experiments extracts obtained from ^{14}C inositol-labelled turions were mixed with extracts from ^3H inositol-labelled frond tissue. Subsequent SAX HPLC did not reveal any peaks unique to frond or turion tissue (results not shown). Furthermore, HPLC of extracts on Adsorbosphere SAX columns with the gradient of [29], although the separation profile was completely different from that obtained with Partisphere SAX HPLC, revealed no difference in the numbers of peaks within the different classes of inositol phosphates between control and ABA-treated tissue.

In some experiments treatment of *Spirodela* cultures with ABA failed to induce turion formation. In these cases analysis of perchloric extracts revealed patterns of peaks on Partisphere SAX HPLC that were no different from control tissue (results not shown). Thus the changes in inositol phosphates detected on ABA treatment seem, at this level of analysis, to be linked to turion induction.

It is against this background of the physiology of turion induction and the large-scale accumulation of phytic acid in *Spirodela polyrhiza* and in plants in general that an analysis of the inositol phosphate content of *Spirodela* and its metabolism must be considered. There is little evidence yet borne out of rigorous enantiomeric or stereochemical analysis that demonstrates that higher plants show the metabolism *in vivo* of inositol phospholipid-derived inositol phosphates that has been extensively characterized in animal systems. In as much as such a metabolism has yet to be demonstrated *in vivo*, perhaps the most striking and established difference between plant and animal inositol phosphate metabolism is the accumulation of phytate deposits in plants to levels typically one to several per cent of seed dry weight or 50–80 % of total seed phosphorus, levels in vast excess of the submillimolar levels typical of animal cells. This is the plant physiological context for the present study.

InsP species

^3H Inositol-labelled fractions taken from the region of the chromatogram in which InsPs are expected to be eluted were desalted, mixed with $\text{D/L-}[^{32}\text{P}]\text{Ins1P}$ and $^3\text{P}\text{Ins2P}$ and re-chromatographed by the method of [18]. Various peaks of ^3H label were resolved, some of which were eluted before D/L-Ins1P and therefore are not *myo*-inositol monophosphates, and one that was eluted precisely with $\text{D/L-}[^{32}\text{P}]\text{Ins1P}$ but before Ins2P , D/L-Ins4P and Ins5P . Similar analyses of fractions purified from charcoal-treated perchloric extracts of $^3\text{P}\text{P}_i$ -labelled tissue revealed the presence of various peaks, some of which were eluted before $\text{D/L-}[^3\text{H}]\text{Ins1P}$, and one that was co-eluted precisely with this standard.

Further analysis of monophosphate isomers was performed on the ^3H -labelled compounds. When presented in a substrate mixture containing an internal standard of $\text{D-}^{14}\text{C}\text{Ins3P}$ to the phytase from *Aspergillus* (Sigma) the D- and/or $\text{L-}^3\text{H}\text{Ins1P}$ obtained from *Spirodela* was metabolized completely to inositol with precisely the same kinetics as the standard (Figure 2b). In a

Figure 2 Metabolism of D- and/or L-[³H]Ins3P obtained from *Spirodela* and D-[¹⁴C]Ins3P by *Aspergillus* phytase

(a) D-[³H]Ins1P was mixed with D-[¹⁴C]Ins3P and incubated with *Aspergillus* phytase in an assay essentially as described below (b). At intervals aliquots were removed, processed as in (b), applied to a Partisphere SAX column and eluted isocratically with 40 mM NaH₂PO₄. The ³H and ¹⁴C contents of the inositol product and InsP substrate were determined by online scintillation counting with a Canberra Packard Radiomatic A500 series flow detector fitted with a 2 ml flow cell, after mixing the HPLC eluate with Canberra Packard Flo Scint IV scintillation cocktail in a ratio of 1:3. (b) A D- and/or L-[³H]Ins3P fraction obtained from [³H]-labelled *Spirodela* was mixed with D-[¹⁴C]Ins3P and incubated in an assay (total volume 220 µl) containing 0.44 mg/ml *Aspergillus* phytase and 20 mM sodium acetate buffer, pH 5.0. At intervals 40 µl aliquots were removed and placed in a boiling water bath for 5 min. To the treated samples was added 100 µl of 5 mM Ins and 10 µl each of 100 µM AMP and 100 µM ADP. The samples were made up to 1 ml with water, applied to a Partisphere SAX column and eluted with either a linear gradient of 0–0.5 M NaH₂PO₄ over 30 min or isocratically with 40 mM NaH₂PO₄, to separate the inositol product from the substrate InsP. Fractions (1.0 min) were collected and counted for ³H and ¹⁴C by dual-label scintillation counting. The results of experiments (a) and (b) are presented as percentage metabolism of InsP to inositol. Symbols: ○, ³H; ●, ¹⁴C. This experiment was performed twice with similar results.

parallel experiment, authentic D-[¹⁴C]Ins3P and D-[³H]Ins1P when combined in a substrate mixture were metabolized with different kinetics (Figure 2a). Although the substrate specificity of the *Aspergillus* phytase for an inositol phosphate substrate is not absolute, the restriction of the number of the potential substrates to two, D-Ins1P and D-Ins3P, simplifies the question of specificity. That the *Spirodela*-derived D- and/or L-[³H]InsP was metabolized with kinetics identical to those of an internal standard of D-[¹⁴C]Ins3P is strong evidence for the identification of the *Spirodela* InsP as D-Ins3P, taking into account the different metabolism of D-[³H]Ins1P and D-[¹⁴C]Ins3P.

InsP₂ species

Several putative InsP₂ species were detected in labelling experiments with [³H]Ins and [¹⁴C]Ins. Partisphere SAX HPLC of

perchloric acid extracts revealed three peaks of ³H-, ¹⁴C- or ³²P-labelled material in the region in which InsP₂ standards were eluted. These labelled peaks were desalted and individually mixed with aliquots of conversely-labelled standards and rechromatographed on Partisphere SAX (results not shown) by the method of [18].

The earlier-eluted compound was co-eluted with Ins(1,4)P₂, the second with D- and/or L-Ins(3,4)P₂, obtained as described below, and the third was co-eluted with Ins(4,5)P₂. The determination of the identities of the [³H]InsP₂s was pursued enzymically and by vigorous periodate oxidation, reduction and dephosphorylation of the putative [³H]InsP₂s.

Vigorous oxidation of the putative D- and/or L-[³H]Ins(1,4)P₂ resulted in the total loss of label as volatiles. This identifies the InsP₂ as a member(s) of the enantiomeric pair D/L-Ins(1,4)P₂ or as the *meso* compound Ins(2,5)P₂. D/L-[³H]Ins(4,5)P₂ and the *meso* compound [³H]Ins(4,6)P₂ also lose their ³H label on vigorous oxidation but all elute substantially later than Ins(1,4)P₂ on this HPLC separation. Ins(2,5)P₂ elutes later than Ins(1,4)P₂ on this HPLC system [for elution of Ins(4,6)P₂ see next section].

The enantiomeric identity of the putative D- and/or L-[³H]Ins(1,4)P₂ was pursued by mild periodate cleavage, reduction and dephosphorylation performed by the method of [30]. The reaction products were separated on Polypore Pb²⁺, yielding a minor peak of iditol and a large peak of unreacted inositol (Figure 3). The generation of iditol by mild treatment with periodate and not glucitol and glycerol, which are the products of mild oxidation of Ins(2,5)P₂, identify the parent InsP₂ as either or both of the enantiomeric pair D/L-Ins(1,4)P₂. The enantiomeric identity of the iditol produced was determined by presenting the iditol as a substrate to L-iditol dehydrogenase (see the Materials and methods section). Subsequent rechromatography of the iditol dehydrogenase-treated sample revealed that under conditions in which approx. 50 % of the unlabelled L-iditol substrate had been metabolized, there was no detectable metabolism of the [³H]iditol (results not shown). This result identifies the [³H]iditol product of mild treatment with periodate as D-[³H]iditol and hence the parent InsP₂ as D-Ins(1,4)P₂.

The second putative [³H]InsP₂ yielded [³H]threitol on vigorous oxidation, reduction and dephosphorylation (results not shown) whereas the same unknown InsP₂ but labelled from [¹⁴C]Ins was co-eluted precisely with a standard of [³H]Ins(3,4)P₂ prepared by treatment of [³H]Ins(1,3,4)P₃ with alkaline phosphatase (Figure 3). The product of treatment of [³H]Ins(1,3,4)P₃ with alkaline phosphatase was eluted after a standard of [³²P]Ins(1,4)P₂. All these observations identify the putative [³H]InsP₂ or [¹⁴C]InsP₂ as D- and/or L-Ins(3,4)P₂. The enantiomerism of this InsP₂ was determined in two further steps. First, the InsP₂, labelled from [³H]Ins, was presented as a substrate to a commercial preparation of phytase (Sigma) from *Aspergillus*, in an assay that contained a trap of 10 mM D/L-Ins3P to limit further breakdown to inositol. The mixture of InsP species produced was resolved on Partisphere SAX eluted isocratically with ammonium acetate solution and the fraction corresponding to D- and/or L-Ins3P desalted by freeze-drying. In a separate experiment the products of phytase treatment were mixed with standards of D/L-[¹⁴C]Ins3P, [¹⁴C]Ins2P and D/L-[¹⁴C]Ins4P and separated by the method of [18]. Two [³H]InsP peaks that were co-eluted precisely with D/L-[¹⁴C]Ins3P and D/L-[¹⁴C]Ins4P were identified (results not shown).

The purified and desalted D- and/or L-[³H]Ins3P, obtained above by phytase treatment of D- and/or L-[³H]Ins(3,4)P₂, was presented as substrate, in an assay containing an internal standard of D-[¹⁴C]Ins3P, to a preparation of the *Aspergillus* phytase. The results of this experiment (not shown) indicate that the [³H]InsP

Figure 3 Identification of InsP_2 s in extracts of *Spirodela*

Fractions corresponding to the three putative InsP_2 peaks shown in Figure 1 were obtained from [^3H] Ins -labelled *Spirodela* by anion-exchange chromatography on Partisphere SAX. (a) A fraction that was co-eluted with an authentic standard of $\text{Ins}(1,4)\text{P}_2$ was desalted and subjected to mild periodate oxidation, reduction and dephosphorylation to yield [^3H]polyols. The sample was mixed with approx. 10 000 d.p.m. of [^{14}C]glucitol and the identities of the [^3H]polyols obtained were determined by HPLC on a Brownlee Polypore Pb^{2+} column at a flow rate of 0.4 ml/min (50 μl injection loop). Fractions (1.0 min) were collected and the ^3H content of a 20 μl aliquot determined by dual-label scintillation counting. The position of elution of unlabelled polyol standards was determined in a parallel HPLC run by measurement of UV absorbance at 200 nm (all steps as described in the Materials and methods section). Symbols: $\circ$, ^3H ; $\bullet$, ^{14}C . The [^3H]iditol obtained was not metabolized when presented to polyol dehydrogenase under conditions in which approx. 50% of the unlabelled L-iditol substrate was converted to products (see text). (b) A pooled InsP_2 fraction corresponding to the second and third peaks of putative [^3H] InsP_2 (Figure 1) was desalted and resolved by Partisphere SAX HPLC according to the method of [18]. The column eluate was split and the radioactivity in a fraction (2%) of the eluate was determined by online scintillation counting in a Canberra Packard Radiomatic A-500 series flow detector fitted with a 0.5 ml flow-cell using Flo-Scint IV (Canberra Packard) scintillation cocktail at a flow rate of 0.38 ml/min. An integration interval of 0.1 min was employed. The remainder of the column eluate was diverted to a fraction collector. (c) An InsP_2 fraction was obtained from [^{14}C] Ins -labelled tissue. A peak fraction that in a separate HPLC run was co-eluted precisely with the first-eluted peak in (b) was mixed with [^3H] $\text{Ins}(3,4)\text{P}_2$ [obtained by alkaline phosphatase treatment of $\text{Ins}(1,3,4)\text{P}_3$] and resolved by Partisphere SAX HPLC according to the method of [18]. Fractions were collected and radioactivity was estimated

obtained on phytase treatment of D- and/or L- $\text{Ins}(3,4)\text{P}_2$ was metabolized with identical kinetics to the internal standard, i.e. that the InsP is the D- $\text{Ins}3\text{P}$ enantiomer and hence that the parent InsP_2 contains a D-3-phosphate. Taken together with the prior derivation of threitol from this compound, the above analyses identify the parent InsP_2 as D- $\text{Ins}(3,4)\text{P}_2$.

The third putative InsP_2 was co-eluted precisely with a standard of [^{32}P] $\text{Ins}(4,5)\text{P}_2$ and was eluted after $\text{Ins}(3,4)\text{P}_2$, identified above, and after ^{32}P -labelled standards of $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(2,4)\text{P}_2$, $\text{Ins}(1,5)\text{P}_2$ and $\text{Ins}(2,5)\text{P}_2$, all prepared from [^{32}P]Ptd $\text{Ins}4\text{P}$ and [^{32}P]Ptd $\text{Ins}(4,5)\text{P}_2$ by deacylation, deglyceration and where appropriate subsequent treatment with alkaline phosphatase, or acid or alkaline hydrolysis. The compound yielded only volatiles on vigorous oxidation, reduction and dephosphorylation. Figure 3 shows the precise co-elution of a [^{14}C] InsP_2 , obtained from [^{14}C] Ins -labelled tissue and that was eluted after $\text{Ins}(3,4)\text{P}_2$ identified above, with a standard of [^3H] $\text{Ins}(4,5)\text{P}_2$ obtained by alkaline phosphatase treatment of [^3H] $\text{Ins}(1,4,5)\text{P}_3$. The elution of this putative InsP_2 after $\text{Ins}(3,4)\text{P}_2$ possibly identifies this compound as either or both of the enantiomeric pair D/L- $\text{Ins}(4,5)\text{P}_2$ or the *meso* compound $\text{Ins}(4,6)\text{P}_2$. *Aspergillus* phytase treatment of [^{32}P] $\text{Ins}(3,4,6)\text{P}_3$, identified by co-elution with [^3H] $\text{Ins}(3,4,6)\text{P}_3$ (see below), yielded a [^{32}P] InsP_2 , presumably $\text{Ins}(4,6)\text{P}_2$, that was co-eluted with authentic $\text{Ins}(4,5)\text{P}_2$ but not with $\text{Ins}(1,4)\text{P}_2$ or $\text{Ins}(3,4)\text{P}_2$. Thus it was not possible to distinguish between the alternative identities of the third putative InsP_2 as either or both of the enantiomeric pair D/L- $\text{Ins}(4,5)\text{P}_2$ or $\text{Ins}(4,6)\text{P}_2$ because all these bisphosphates lose their ^3H label even on mild cleavage with periodate. Alkaline hydrolysis of the [^3H] InsP_2 yielded products that when mixed with acid-hydrolysed [^{14}C] $\text{Ins}3\text{P}$ were co-eluted with D/L- ^{14}C $\text{Ins}4\text{P}$ and after D/L- ^{14}C $\text{Ins}3\text{P}$ and [^{14}C] $\text{Ins}2\text{P}$ (results not shown). The failure to observe products that were co-eluted with either $\text{Ins}3\text{P}$ and $\text{Ins}2\text{P}$ further confirms that the parent InsP_2 could contain phosphates in the 4-, 5- and/or 6- positions only.

However, when presented as co-substrate with [^{32}P] $\text{Ins}(4,5)\text{P}_2$ to human erythrocyte ghosts [14] under conditions designed to remove phosphates selectively from the 5-position of inositol phosphates, the unknown ^3H -labelled compound was metabolized with different kinetics to those of [^{32}P] $\text{Ins}(4,5)\text{P}_2$ (results not shown). Thus when 10% of the [^{32}P]Ptd $\text{Ins}(4,5)\text{P}_2$ had been converted to InsP and P_i , the extent of metabolism of the unknown to product(s) with the chromatographic properties of an inositol monophosphate that was eluted marginally later than AMP and before P_i on Partisphere SAX was less than 1.5%.

It is not possible from the above to identify conclusively the unknown ^3H -labelled compound as an inositol phosphate. The pathways of inositol metabolism in plants are not restricted to inositol phosphates and include among their intermediates products of inositol oxidation, sugar nucleotides and cell wall

by dual-label scintillation counting. (All steps were as described in the Materials and methods section.) Symbols: $\circ$, ^3H ; $\bullet$, ^{14}C . (d) An InsP_2 fraction was obtained from [^{14}C] Ins -labelled tissue. An aliquot of a peak fraction that in a separate HPLC run was co-eluted precisely with the second-eluted peak of (b) was mixed with [^3H] $\text{Ins}(4,5)\text{P}_2$ [obtained by treatment of $\text{Ins}(1,4,5)\text{P}_3$ with alkaline phosphatase] and resolved by Partisphere SAX HPLC according to the method of [18]. Radioactivity was determined by online scintillation counting of the total column eluate in a Canberra Packard Radiomatic A-500 series flow detector fitted with a 2.0 ml flow-cell using Flo-Scint IV (Canberra Packard) scintillation cocktail at a flow rate of 3 ml/min. An integration interval of 0.1 min was employed. (All steps were as described in the Materials and methods section.) Symbols: $\circ$, ^3H ; $\bullet$, ^{14}C . In (b), (c) and (d) the retention times are reported relative to an internal ADP marker used to monitor the chromatography in the individual HPLC runs. In (b) and (d) the data are presented as the means of pairs of adjacent data points to improve the clarity of the figure.

Figure 4 Resolution of [^3H]Ins P_3 in extracts of *Spirodela*

A perchloric acid extract derived from [^3H]Ins-labelled *Spirodela* was mixed with acid-hydrolysed [^{32}P]Ins(1,4,5) P_3 , applied to a Partisphere SAX column and eluted with a gradient of NaH_2PO_4 as used to separate the different classes of inositol phosphates and as described in the Materials and methods section. The ^3H and ^{32}P contents of the column eluate were determined by online scintillation counting with a Canberra Packard Radiomatic A500 series flow detector fitted with a 0.5 ml flow cell after mixing the HPLC eluate with Canberra Packard Flo Scint IV scintillation cocktail. Scintillation events were counted with an integration interval of 6 s. The figure shows data corresponding to the time window in which Ins P_3 s were eluted. The labels refer to the positions of peaks of ^{32}P standards. Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P . Similar results have been obtained on at least four occasions from different preparations of Ins P_3 from ^3H - or ^{32}P -labelled tissue with conversely labelled standards of Ins(1,4,5) P_3 and Ins(2,4,5) P_3 .

components [11]. The cell walls of turions of *Spirodela polyrhiza* are heavily thickened [13,27] and it is not unreasonable to assume that inositol is incorporated into components of the cell wall during turion development. Thus Roberts and Loewus [9] showed, in other members of the Lemnaceae, that inositol was incorporated into cell wall components, and Longland et al. demonstrated [31] that glucuronic acid, the oxidation product of inositol, was heavily incorporated into cell wall components in *Spirodela polyrhiza* when turions were induced by ABA treatment. The chemical identity of the third peak therefore remains to be determined. The foregoing therefore adds a cautionary note to analyses of plant inositol phosphates where there is a reliance solely on chromatography, usually by reference to only one or two inositol phosphate standards, to identify endogenous inositol bisphosphates.

Ins P_3 species

Two separate peaks of ^3H label were detected in the Ins P_3 region of SAX chromatograms of ^3H -labelled perchloric acid extracts. The earliest, a minor peak detected in some but not all extracts, was co-eluted with [^{32}P]Ins(1,4,5) P_3 prepared by deacylation and deglyceration of [^{32}P]PtdIns(4,5) P_2 (Figure 4). The identity of this peak has not been determined but preliminary analysis (results not shown) indicates that Ins(1,4,5) P_3 , if present, constitutes only a small fraction of this peak.

The principal [^3H]Ins P_3 peak was eluted after [^{32}P]Ins(1,4,5) P_3 and with [^{32}P]Ins(2,4,5) P_3 , obtained from Ins(1,4,5) P_3 by acid hydrolysis, on SAX columns (Figure 4).

Periodate oxidation, reduction and dephosphorylation of this peak yielded a [^3H]polyol that was eluted in the position of iditol as determined in a parallel HPLC run (results not shown). The [^3H]polyol was presented as a substrate under first-order conditions to L-iditol dehydrogenase [16]. The reaction was stopped before completion and the products analysed by Polypore Pb $^{2+}$

Figure 5 Oxidation of [^3H]iditol, derived from *Spirodela* [^3H]Ins P_3 , by L-iditol dehydrogenase

The [^3H]iditol derived from the major *Spirodela* [^3H]Ins P_3 was presented as a substrate under first-order conditions to L-iditol dehydrogenase. The reaction was stopped by boiling for 3 min, the products were desalted on Amberlite MB-3 resin and freeze-dried. The products were taken up in a small volume of water and reappplied to a Brownlee Polypore Pb $^{2+}$ column (50 μl injection loop) and eluted at a flow rate of 0.4 ml/min. Fractions (1.0 min) were collected and the ^3H content determined by scintillation counting. The positions of elution of unlabelled polyol and sorbose standards were determined in a parallel HPLC run by measurement of UV absorbance at 200 nm (all steps as described in the Materials and methods section). The progress of oxidation of the [^3H]iditol was monitored by measuring the absorbance at 340 nm and is shown in the inset. This experiment was performed twice with similar results.

HPLC (Figure 5). Two ^3H -labelled peaks were identified in the reaction products, one which was eluted before inositol in the position of sorbose, the product of L-iditol oxidation by L-iditol dehydrogenase (the elution position of which was determined in a parallel HPLC run), and a second peak of unreacted substrate [^3H]iditol. Comparison of the extent of oxidation of unlabelled L-iditol (obtained by fitting the progress of reaction curve, A_{340} , to an equation describing a first-order reaction and extrapolation to completion) with the oxidation of the [^3H]iditol of unknown isomerism gave estimates for the extents of reaction of 38 % and 30 % respectively. As the two values are in good agreement and within the limits of accuracy for the experimental techniques we conclude that the [^3H]polyol of unknown isomerism is exclusively the L-isomer and that the parent [^3H]Ins P_3 must be Ins(3,4,6) P_3 .

Ins P_4 species

Ins P_4 -containing fractions obtained from perchloric acid extracts were desalted. [^3H]Ins P_4 was mixed with approximately 600 d.p.m. of [^{32}P]Ins(3,4,5,6) P_4 , 600 d.p.m. of [^{32}P]Ins(1,3,4,5) P_4 and 350 d.p.m. of [^{32}P]Ins(1,3,4,6) P_4 and rechromatographed (see the Materials and methods section) on a gradient designed [16] to separate Ins P_4 isomers (Figure 6). The order of elution of these standards on Partisphere SAX columns was Ins(3,4,5,6) P_4 , then Ins(1,3,4,5) P_4 , followed by Ins(1,3,4,6) P_4 . The results show that a single peak of [^3H]Ins P_4 was obtained from perchloric extracts of *Spirodela* and this peak was co-eluted precisely with

Figure 6 Resolution of $[^3\text{H}]\text{InsP}_4$ s from extracts of *Spirodela*

A $[^3\text{H}]\text{InsP}_4$ fraction from *Spirodela* was mixed with $[^{32}\text{P}]\text{Ins}(3,4,5,6)\text{P}_4$, $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4,6)\text{P}_4$, applied to a Partisphere SAX column and eluted. Fractions (0.6 min) were collected and counted for ^3H and ^{32}P by dual-label scintillation counting (all steps as described in the Materials and Methods section). The labels refer to the positions of peaks of ^{32}P standards. Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P . Elution of the *Spirodela* InsP_4 before $\text{Ins}(1,3,4,5)\text{P}_4$ has been demonstrated on at least three occasions.

the first-eluted of the $[^{32}\text{P}]\text{InsP}_4$ standards. The elution of the $[^3\text{H}]\text{InsP}_4$ with $[^{32}\text{P}]\text{Ins}(3,4,5,6)\text{P}_4$ also excludes D/L- $\text{Ins}(1,2,3,4)\text{P}_4$, $\text{Ins}(1,2,5,6)\text{P}_4$ and $\text{Ins}(1,2,4,6)\text{P}_4$ as potential identities for the $[^3\text{H}]\text{InsP}_4$, as all these isomers were eluted after $\text{Ins}(1,3,4,5)\text{P}_4$ on Partisphere SAX [16]. The identity of the $[^3\text{H}]\text{InsP}_4$ was pursued further by periodate oxidation, reduction and dephosphorylation to produce a $[^3\text{H}]\text{polyol}$, which was resolved on Polypore Pb^{2+} HPLC (results not shown). The $[^3\text{H}]\text{polyol}$ was eluted in the precise position of unlabelled iditol, which was identified in a mixture of unlabelled polyols in a parallel HPLC run. Presentation of the $[^3\text{H}]\text{polyol}$ to L-iditol dehydrogenase and HPLC of the reaction products yielded (Figure 7), in addition to unreacted iditol, a major ^3H -labelled product that was eluted before inositol in the position of sorbose. Good agreement was obtained between estimates of the extent of reaction of the $[^3\text{H}]\text{iditol}$ of unknown isomerism (88%), disregarding the two minor and unidentified peaks, and the extent of the metabolism of L-iditol (84%) determined by fitting the reaction curve to a first-order equation and extrapolating to completion. This result identifies the $[^3\text{H}]\text{InsP}_4$ as $\text{Ins}(3,4,5,6)\text{P}_4$.

InsP_5 species

InsP_5 -containing fractions obtained by SAX HPLC of extracts from ^3H -labelled tissue were desalted and an aliquot was mixed with an aliquot of a $[^{32}\text{P}]\text{InsP}_5$ fraction obtained from germinating mung beans labelled for 60 h, extracted, HPLC-purified and desalted by the method of [16]. The mixed $[^3\text{H}]\text{InsP}_5$ s and $[^{32}\text{P}]\text{InsP}_5$ s were resolved on Partisphere SAX by using a system from Stephens [16]. At best four InsP_5 species can be resolved on this system; in order of elution they are $\text{Ins}(1,3,4,5,6)\text{P}_5$ (2-OH), D/L- $\text{Ins}(1,2,3,4,5)\text{P}_5$ (4/6-OH), $\text{Ins}(1,2,3,4,6)\text{P}_5$ (5-OH) and D/L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ (1/3-OH), but $\text{Ins}(1,2,3,4,6)\text{P}_5$ and D/L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ were almost co-eluted [16,19].

A SAX HPLC separation of a $[^3\text{H}]\text{InsP}_5$ fraction from *Spirodela*

Figure 7 Oxidation of $[^3\text{H}]\text{iditol}$, derived from *Spirodela* $[^3\text{H}]\text{InsP}_4$, by L-iditol dehydrogenase

The $[^3\text{H}]\text{iditol}$ derived from *Spirodela* $[^3\text{H}]\text{InsP}_4$ was presented as a substrate under first-order conditions to L-iditol dehydrogenase. The reaction was stopped by boiling for 3 min, the products were desalted on Amberlite MB-3 resin and freeze-dried. The products were taken up in a small volume of water and reappplied to a Brownlee Polypore Pb^{2+} column (50 μl injection loop) and eluted at a flow rate of 0.4 ml/min. Fractions (1.0 min) were collected and the ^3H content was determined by scintillation counting. The positions of elution of unlabelled polyol and sorbose standards were determined in a parallel HPLC run by measurement of UV absorbance at 200 nm (all steps as described in the Materials and Methods section). The progress of oxidation of the $[^3\text{H}]\text{iditol}$ was monitored by measuring the absorbance at 340 nm and is shown in the inset. This experiment was performed twice with similar results.

mixed with the $[^{32}\text{P}]\text{InsP}_5$ fraction from mung beans is shown in Figure 8(a). A single peak of ^3H radioactivity was co-eluted precisely with a small peak of ^{32}P radioactivity and before two major peaks of ^{32}P radioactivity. The two principal ^{32}P -labelled InsP_5 species obtained from germinating mung beans are D- and/or L- $\text{Ins}(1,2,3,4,5)\text{P}_5$ and D- and L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ [16,19]. Thus it is likely that the minor peak of ^{32}P label and the major peak of ^3H label with which it is co-eluted are $\text{Ins}(1,3,4,5,6)\text{P}_5$. This isomer was not described by Stephens and coworkers, and its identification here gives significance *in vivo* to the early purification [5] of an enzyme activity from mung bean capable of catalysing the interconversion of InsP_6 with ATP and $\text{Ins}(1,3,4,5,6)\text{P}_5$. Confirmation of the identity of the $[^3\text{H}]\text{InsP}_5$ as $\text{Ins}(1,3,4,5,6)\text{P}_5$ was obtained by acid-catalysed phosphate migration (see the Materials and Methods section). Limited acid-treatment of $\text{Ins}(1,3,4,5,6)\text{P}_5$ (2-OH) should result in phosphate migration between the 1 and 2 and the 2 and 3 positions, generating a peak of D/L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ (1/3-OH). On SAX HPLC of the acid-treated $[^3\text{H}]\text{InsP}_5$ (Figure 8b) a second peak of ^3H radioactivity was detected that was co-eluted with the last-eluted peak of ^{32}P -radioactivity, i.e. with D- and L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ (1/3-OH).

Further confirmation of the identity of the $[^3\text{H}]\text{InsP}_5$ was obtained by treatment with erythrocyte ghosts (see the Materials and Methods section) of the $[^3\text{H}]\text{InsP}_5$ in the presence of 5 mM MgCl_2 and the absence of EDTA. The products of this treatment were mixed with D- and/or L- $[^{32}\text{P}]\text{Ins}(1,2,3,4)\text{P}_4$ [obtained from germinating mung beans and purified from $[^{32}\text{P}]\text{Ins}(1,2,5,6)\text{P}_4$, the other principal isomer identified in this tissue] and resolved

Figure 8 Identification of $[^3\text{H}]\text{Ins}(1,3,4,5,6)\text{P}_5$ in extracts of *Spirodela*

The major $[^3\text{H}]\text{InsP}_5$ fraction from *Spirodela* was desalted and (a) mixed with a $[^{32}\text{P}]\text{InsP}_5$ fraction obtained from germinating mung bean seedlings or (b) acid-hydrolysed, desalted, mixed with an aliquot of the same $[^{32}\text{P}]\text{InsP}_5$ fraction and separately applied to a Partisphere SAX HPLC column. Fractions (0.5 or 0.4 min) were collected and counted for ^3H and ^{32}P by dual-label scintillation counting (all steps as described in the Materials and methods section). Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P .

on a gradient designed to separate InsP_4 isomers [16]. A single peak of ^3H radioactivity was eluted fractionally before and overlapping D- and/or L- $[^{32}\text{P}]\text{Ins}(1,2,3,4)\text{P}_4$ (results not shown) and, in a separate HPLC run, after $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ (Figure 9b). Although an erythrocyte ghost $\text{Ins}(1,3,4,5,6)\text{P}_5$ 5-phosphatase activity has to our knowledge not yet been identified, the generation of a product that is eluted after $\text{Ins}(1,3,4,5)\text{P}_4$ and fractionally before D- and/or L- $\text{Ins}(1,2,3,4)\text{P}_4$ is good evidence for the generation of an $\text{Ins}(1,3,4,6)\text{P}_4$ product from $\text{Ins}(1,3,4,5,6)\text{P}_5$. Note that $\text{Ins}(1,3,4,6)\text{P}_4$ is eluted between $\text{Ins}(1,3,4,5)\text{P}_4$ and D/L- $\text{Ins}(1,2,3,4)\text{P}_4$ on Partisphere SAX columns [16]. Under a different set of experimental conditions (1 mM MgCl_2) human erythrocyte ghost treatment of the InsP_5 yielded a product that was eluted before $\text{Ins}(1,3,4,5)\text{P}_4$ and was co-eluted precisely with $[^3\text{H}]\text{Ins}(3,4,5,6)\text{P}_4$ (Figure 9a), a result consistent with the action of the well documented $\text{Ins}(1,3,4,5,6)\text{P}_5$ 3-phosphatase activity of the erythrocyte membrane and one that further confirms the $\text{Ins}(1,3,4,5,6)\text{P}_5$ structure of the parent compound.

In a very few experiments traces were detected of a second InsP_5 , which constituted typically less than 0.5% of the label in $\text{Ins}(1,3,4,5,6)\text{P}_5$ and was eluted after that compound on Partisphere SAX columns. The identification of this compound was

Figure 9 Treatment of a $[^3\text{H}]\text{InsP}_5$ from *Spirodela* with erythrocyte ghosts

(a) An InsP_5 fraction obtained from $^3\text{H}/^{32}\text{P}$ -labelled *Spirodela* was resolved by Partisphere SAX HPLC and desalted. The preparation of InsP_5 was dephosphorylated with human erythrocyte ghosts in the presence of 1 mM MgCl_2 . The products were resolved on Partisphere SAX HPLC by using a gradient designed to separate InsP_4 species. A single peak of InsP_4 was obtained. An aliquot of this peak containing approx. 1000 d.p.m. each of ^3H and ^{32}P radioactivity was mixed with approx. 5000 d.p.m. of $[^3\text{H}]\text{Ins}(3,4,5,6)\text{P}_4$ and 5000 d.p.m. of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ and resolved on Partisphere SAX HPLC by using a gradient designed to separate InsP_4 species. Fractions were collected and counted for ^3H and ^{32}P (all steps as described in the Materials and methods section). Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P . Only that region of the chromatogram in which InsP_4 species were eluted is shown. (b) An InsP_5 fraction obtained from a separate $^3\text{H}/^{32}\text{P}$ -labelling experiment from that described above was dephosphorylated with human erythrocyte ghosts in the presence of 5 mM MgCl_2 . The products were resolved on Partisphere SAX HPLC by using a gradient designed to separate InsP_4 species. A single peak of InsP_4 was obtained. An aliquot of this peak containing approx. 700 d.p.m. of ^3H and 1400 d.p.m. of ^{32}P radioactivity was mixed with approx. 2100 d.p.m. of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ and resolved on Partisphere SAX HPLC by using a gradient designed to separate InsP_4 species. Fractions were collected and counted for ^3H and ^{32}P (all steps as described in the Materials and methods section). Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P . Only that region of the chromatogram in which InsP_4 species were eluted is shown. Similar experiments to these using single- or dual-labelled preparations of InsP_5 and conversely labelled standards were performed on at least three occasions with identical results.

pursued by $[^{14}\text{C}]\text{inositol}$ labelling of intact abscised turions. Partisphere SAX HPLC of a purified and desalted fraction with an acid-hydrolysed standard of $[^3\text{H}]\text{Ins}(1,3,4,5,6)\text{P}_5$ showed that the ^{14}C -labelled compound was eluted after the traces of D/L- $\text{Ins}(1,2,3,4,5)\text{P}_5$ generated by this procedure and with the major product D/L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ (results not shown). This leaves the possibility that the ^{14}C -labelled compound may be either D- and/or L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ and/or $\text{Ins}(1,2,3,4,6)\text{P}_5$. The former possibility was confirmed by HPLC of the compound and the same ^3H standards on Adsorbosphere SAX by the method of [29]. In these circumstances the ^{14}C -labelled compound was eluted after D/L- $\text{Ins}(1,2,3,4,5)\text{P}_5$ and was co-eluted precisely with D/L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ (Figure 10). This result, taken together with the former result on Partisphere SAX HPLC, leaves D- and/or L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ as the only possible structure. This compound is not resolved from D/L- $\text{Ins}(1,3,4,5,6)\text{P}_5$ on Adsorbosphere SAX columns [29].

Figure 10 Identification of D- and/or L-[^{14}C]Ins(1,2,4,5,6) P_5 in intact abscised [^{14}C]Ins-labelled *Spirodela* turions

A [^{14}C]Ins P_5 fraction that was eluted after Ins(1,3,4,5,6) P_5 on Partisphere SAX HPLC was obtained from intact abscised turions of *Spirodela* and desalted. The Ins P_5 was mixed with a sample of acid-hydrolysed [^3H]Ins(1,3,4,5,6) P_5 and applied to an Adsorbosphere SAX HPLC column and eluted by the method described in [29]. Fractions were collected and counted for ^3H and ^{14}C by dual-label scintillation counting (all steps as described in the Materials and methods section). Symbols: $\circ$, ^3H ; $\bullet$, ^{14}C .

Ins P_6

The most polar fraction to be eluted under SAX HPLC, labelled from both [^3H]Ins and [^{32}P]orthophosphate, was putatively identified as Ins P_6 . The identity of this compound was confirmed by mixing an aliquot of the desalted ^3H -labelled peak with an aliquot of desalted ^{32}P -labelled Ins P_6 obtained from [^{32}P]P $_i$ -labelled germinating mung beans (all steps as described in [16]) and rechromatography of the sample on Partisphere SAX HPLC (results not shown).

DISCUSSION

With the exception of studies on avian erythrocytes [17,22,23,30], *Dictyostelium* [1], WRK1 rat mammary tumour cells [32,33], and T5-1 cells [34,35], the analysis of inositol phosphates in a given cell type has usually been limited to those directly implicated in signal transduction. Just as the complexity of inositol phosphate-mediated signal transduction mechanisms has increased to accommodate inositol tetrakisphosphate metabolism and rather more recently novel 3-phosphorylated lipids, there has been an increasing interest in inositol pentakisphosphate and hexakisphosphate metabolism. Despite the growing realization of the ubiquity of inositol hexakisphosphate in mammalian as well as plant cells and the implied possibility of metabolic links between second messenger and inositol hexakisphosphate metabolism, few studies have attempted to identify all of the inositol phosphates in a given cell type.

The situation in plants is particularly unclear and is compounded by an almost complete absence of rigorous identifications of inositol phosphates. We have previously characterized the pathways of synthesis of the 3-, 4- and 5-phosphorylated phosphatidylinositols in the aquatic plant *Spirodela polyrrhiza* [14,25] and have now turned our attention to the range of inositol phosphates that can be identified *in vivo* in *Spirodela*.

Apart from Ins P_6 and those inositol phosphates that appear, in animal cell systems at least (see, for example, [36]), to be products

of second messenger metabolism *in vivo*, namely Ins3 P , Ins4 P , Ins(1,4) P_2 , Ins(3,4) P_2 , Ins(4,5) P_2 , Ins(1,3,4) P_3 , Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , the other inositol phosphate species identified in *Spirodela* have little or no precedent in the plant kingdom. It is tempting to speculate that the other inositol phosphates identified, namely Ins(3,4,6) P_3 , Ins(3,4,5,6) P_4 and Ins(1,3,4,5,6) P_5 , are intermediates in the synthetic sequence to Ins P_6 . A not inconsiderable body of circumstantial evidence supports this proposition.

First, the existence principally of single major species of inositol polyphosphate at each level of phosphorylation above Ins P_3 argues strongly that these inositol polyphosphates are not products of Ins P_6 breakdown because the phytases of plant origin are not entirely specific in their attack on Ins P_6 [37], leading to a diversity of products at subsequent levels of dephosphorylation. More specifically, the preferred site of attack of plant phytases at the D-4 position of Ins P_6 to yield Ins(1,2,3,5,6) P_5 is inconsistent with the identification of Ins(1,3,4,5,6) P_5 as the principal Ins P_5 isomer. Indeed, Ins2 P is the penultimate product *in vitro* of phytase action on Ins P_6 [37]. Perhaps significantly, Ins2 P was not detected in *Spirodela*. Moreover, these intermediates were labelled at a developmental stage when massive synthesis and accumulation of Ins P_6 was occurring (Figure 1).

Secondly, although Ins(3,4,6) P_3 has been identified in avian erythrocytes [17], WRK1 rat mammary tumour cells [33] and *Dictyostelium* [1], the only metabolic role yet identified for this inositol phosphate appears to be as an intermediate in Ins P_6 synthesis [1].

Thirdly and similarly, although Ins(3,4,5,6) P_4 has been detected in WRK1 rat mammary tumour cells [33], a rat pancreatoma cell line [29] and bovine adrenal glomerulosa cells [38], and its levels are stimulated by agonists to a greater extent than its enantiomer Ins(1,4,5,6) P_4 , the low to undetectable level of labelling of Ins(1,4,5) P_3 and Ins(4,5) P_2 in *Spirodela* and plant systems in general seems to preclude a second messenger source for the Ins(3,4,5,6) P_4 identified in *Spirodela*. Moreover, that the only Ins P_4 detected in *Spirodela* is the Ins(3,4,5,6) P_4 isomer cannot be rationalized by the simple one-step metabolism of Ins(1,4,5) P_3 . Ins(1,4,5) P_3 has only recently been rigorously identified in plants [39] and little is known of the routes of metabolism of this compound *in vivo* in higher plants. Thus the only other Ins P_3 identified in plants with any rigour is Ins(3,4,6) P_3 described above. In this context the demonstration in pea root homogenates [40] of an enzyme activity capable of phosphorylating Ins(1,4,5) P_3 in the 6-position deserves mention, although the use of only one substrate makes it difficult to reach any firm conclusions about its specificity and therefore its likely role *in vivo*.

Fourthly, although several Ins P_5 s have been detected in *Dictyostelium* Ins(1,3,4,5,6) P_5 is the *de novo* precursor of Ins P_6 synthesis in that organism. Perhaps similarly, an enzyme activity capable of transferring a phosphate from ATP to Ins(1,3,4,5,6) P_5 in a reversible manner has been purified from mung beans [5], though unfortunately again the specificity of the activity towards other substrates was not determined.

In the following paper [12] we present direct evidence of the order in which individual phosphates have been added to the inositol moiety of Ins P_6 and in doing so describe the pathway of synthesis of Ins P_6 .

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