Metabolic evidence for the order of addition of individual phosphate esters to the *myo*-inositol moiety of inositol hexakisphosphate in the duckweed *Spirodela polyrhiza* L.

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The aquatic monocotyledonous plant *Spirodela polyrhiza* was labelled with [^{32}P]P_i for short periods under non-equilibrium conditions. An InsP₆ fraction was obtained and dissected by using enantiospecific (enzymic) and non-enantiospecific (chemical) means to determine the relative labelling of individual phosphate substituents on the inositol ring of InsP₆. Phosphates in positions D-1, -2, -3, -4, -5 and -6 contained approx. 21 %, 32–39 %, 9–10 %, 14–16 %, 19–23 % and 16–18 % of the label respectively. We conclude from the foregoing, together with identities [described in the preceding paper, Brearley and Hanke

(1996) Biochem. J. 314, 215–225] of inositol phosphates found in this plant at a developmental stage associated with massive accumulation of InsP₆, that synthesis of InsP₆ from *myo*-inositol proceeds according to the sequence Ins3P → Ins(3,4)P₂ → Ins(3,4,6)P₃ → Ins(3,4,5,6)P₄ → Ins(1,3,4,5,6)P₅ → InsP₆ in *Spirodela polyrhiza*. These results represent the first description of the synthetic sequence to InsP₆ in the plant kingdom and the only comprehensive description of endogenous inositol phosphates in any plant tissue. The sequence described differs from that reported in the slime mould *Dictyostelium discoideum*.

INTRODUCTION

In the preceding paper [1] we have undertaken a rigorous analysis of the individual inositol phosphates labelled from inositol in the duckweed *Spirodela polyrhiza*. A considerable body of circumstantial evidence suggests that certain of these inositol phosphates represent intermediates on the synthetic pathway between *myo*-inositol and InsP₆.

An earlier study [2] of InsP₆ synthesis in the slime mould *Dictyostelium discoideum* established the individual synthetic steps in the metabolic sequence by an approach *in vitro* whereby all of the putative intermediates, most of which had been characterized structurally in extracts of inositol-labelled *Dictyostelium discoideum*, were presented to homogenates of *Dictyostelium discoideum* in the presence of an ATP-regenerating system and were shown to be converted to more highly phosphorylated forms in the putative sequence.

Here we set out to establish, in the aquatic plant *Spirodela polyrhiza* L., the order in which individual phosphates are esterified on to inositol to give InsP₆. We have employed the strategy of [3], which we previously used in an analysis of inositol phospholipid metabolism in *Spirodela polyrhiza* [4], to determine, from the [^{32}P]P_i labelling of individual phosphates relative to each other, the sequence in which each of these phosphates is added to inositol.

MATERIALS AND METHODS

Reagents

myo-[2- ^3H]Inositol and [^{32}P]P_i (carrier-free) were obtained from Amersham International (Amersham, Bucks., U.K.). Phytase from *Aspergillus ficuum* was obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.).

Tissue

The aquatic monocotyledonous plant *Spirodela polyrhiza* L. was maintained in axenic culture by the method of [5]. For labelling experiments the plant growth regulator abscisic acid was included in the culture medium at a concentration of 0.3 μM .

Tissue extraction

After the plants had been labelled they were washed and then cooled in liquid nitrogen, ground in a liquid-nitrogen-cooled mortar and pestle and extracted with 0.7–1.0 ml of 3.5 % (w/v) perchloric acid. Cell debris was pelleted by centrifugation for 3 min at 13 000 g_{max} in a refrigerated Microfuge. The supernatant was neutralized to pH 6–7 with 2 M KOH, 100 mM Mes, 10 mM EDTA and held on ice for approx. 15 min before centrifugation for 5 min in a refrigerated bench centrifuge at 3000 g_{max} . The supernatant was removed and applied to a 25 cm Partisphere strong-anion-exchange (SAX) column and eluted as described in the preceding paper [1]. In some experiments, before neutralization, perchloric acid extracts were treated with charcoal essentially as described [6] to remove most of the nucleotide fraction.

Radiolabelling

Plants were labelled with [^3H]inositol or [^{32}P]P_i essentially as described previously [4]. In ^{32}P -labelling experiments, plants (150–300 mg) were typically inoculated into 2 ml of half-strength Hutner's medium containing 2 mCi of [^{32}P]P_i and incubated at 25 °C for 30–40 min.

Rationale of short-term [^{32}P]P_i labelling

The theoretical basis of the method described in detail [3] is that

Abbreviations used: we have used the nomenclature of [10] 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.

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during a short period of labelling with [^{32}P]P_i the individual phosphates in a given inositol phosphate will be far removed from isotopic equilibrium with ATP and more specifically its γ -phosphate. Thus in a biosynthetic process in which phosphates are added in sequence, the phosphate added last in the sequence will have been added at a point when the specific radioactivity of the parent pool of ATP is greater than that at the time of addition of the prior phosphates and thus will be more strongly labelled than its counterparts.

Analysis of the distribution of label between individual phosphates of two apparent 'neighbours' in a putative metabolic sequence may confirm or deny that the 'neighbours' are metabolically related to each other; however, without additional information, namely the absolute specific radioactivities of the two molecules, the short-term labelling data alone cannot distinguish between a synthetic and a degradative relationship between the two intermediates.

We have restricted ourselves here to an analysis of the distribution of label in individual phosphates of InsP₆. As InsP₆ accumulating in developing turions represents the endpoint of a metabolic sequence (there is at present no evidence for inositol pyrophosphates in *Spirodela polyrhiza*), there can be no doubt that we are looking at the order of addition of phosphates to inositol to yield InsP₆. We have previously shown [4] that the specific radioactivity of the γ -phosphate of ATP and of the ATP pool as a whole increases progressively over a 40 min period of labelling with [^{32}P]P_i and may well continue to do so for some time after.

Dissection of InsP₆

It is of crucial importance to an analysis of labelling of individual phosphates to be able to remove in a specific manner individual phosphates from defined positions of an inositol polyphosphate, to separate the products chromatographically and to identify them unambiguously. Dissection of inositol phospholipids is relatively straightforward and can be performed at the level of the water-soluble deacylation products, or subsequently at that of the deglycerated products, by use of erythrocyte ghost inositol phosphatase activities [4,7–9] or with alkaline phosphatase and inositol phosphate phosphatase activities from mammalian sources [7,9]. Where such analyses have been applied to higher inositol polyphosphates [3], the identification of the numerous products of dissection of all levels of phosphorylation is considerably more difficult and has been achieved by conventional chromatographic means coupled with an analysis of the polyol products obtained from ^3H and ^{32}P dual-labelled starting materials.

We have restricted the dissection of InsP₆ to an analysis of the product InsP₅ species. Removal of a single phosphate from each of the six positions of InsP₆ yields six InsP₅ species, which form two pairs of enantiomers and two separate *meso*-compounds. These six compounds can be resolved at best into four distinct peaks of Ins(1,3,4,5,6)P₅ [InsP₅ (2-OH)], D/L-Ins(1,2,3,4,5)P₅ [InsP₅ (4/6-OH)], D/L-Ins(1,2,4,5,6)P₅ [InsP₅ (1/3-OH)] and Ins(1,2,3,4,6)P₅ [InsP₅ (5-OH)] by conventional non-chiral chromatography on a combination of Partisphere SAX and Partisphere WAX HPLC columns [6,10]. The use of two columns is required because neither column alone completely separates all four species. Thus, for example, InsP₅ (5-OH) is poorly resolved from InsP₅ (1/3-OH) on Partisphere SAX but the two can be resolved on Partisphere WAX. Alternatively, resolution of InsP₅ (5-OH) from InsP₅ (1/3-OH) can be achieved on Adsorbosphere SAX columns [11].

Alkaline hydrolysis of InsP₆ and HPLC of products

Phosphates were removed from InsP₆ by limited alkaline hydrolysis performed by the method of [6]. [^3H]InsP₆ and [^{32}P]InsP₆ were dissolved in 0.5 ml of 50 mM Na₂HPO₄ (pH 10.5 with NaOH at 25 °C) and heated at 120 °C for 3 h. The products were adjusted to pH 6–7 with formic acid and subjected to HPLC on either Partisphere SAX (see preceding paper [1]) or on a 25 cm Adsorbosphere SAX column [11] purchased from Alltech Ltd. (Carnforth, U.K.). The column was eluted with a gradient derived from buffers A (water) and B (1 M NH₄H₂PO₄, pH 3.35, with H₃PO₄, at 25 °C) at a flow rate of 1 ml/min: 0 min, 0 % B; 120 min, 100 % B; 140 min, 100 % B.

Alkaline hydrolysis yields a mixture of all six InsP₅ species: the two *meso*-compounds InsP₅ (2-OH) and InsP₅ (5-OH), and racemic mixtures of InsP₅ (4/6-OH) and InsP₅ (1/3-OH). Alkaline hydrolysis, although clearly of a non-enantiospecific nature, is, perhaps surprisingly, stereospecific. InsP₅ (1/3-OH) is the predominant product of alkaline hydrolysis of InsP₆ [10]. This aside, the ^{32}P label lost in generating a racemic InsP₅ by alkaline hydrolysis represents the arithmetical average of the radioactivity associated with equal chemical amounts of each of the two enantiomeric phosphate ester moieties. Thus for a peak of racemic InsP₅ (4/6-OH) with an associated loss of, for example, 15 % of the ^{32}P label of the parent InsP₆, the value of 15 % represents label lost on removal of equal amounts of the 4- and 6-phosphates. This also gives the upper and lower limits of labelling of, for example, the 4- and 6-phosphates: 0 % and 30 %, or vice versa.

Such non-enantiospecific attack yields no information about the relative strength of labelling in structurally equivalent positions 1/3 and 4/6. Such information may be obtained by the use of enzymes with stereospecific tendencies. Of great potential value in this respect are the commercial preparations of phytases from *Aspergillus ficuum* and wheat bran. The principal product of attack on InsP₆ by the phytase from *Aspergillus* is InsP₅ (3-OH) and a minor product is InsP₅ (6-OH), with little or no InsP₅ (4-OH) [6,12]. Alternatively, InsP₅ (4-OH) can be obtained as the major product of attack by wheat bran phytase on InsP₆ [6,13,14].

Phytase treatment of InsP₆

We have used the *Aspergillus* phytase essentially as described [6]. [^3H]InsP₆ and [^{32}P]InsP₆ were typically incubated at 37 °C for 1 h with *Aspergillus* phytase (0.001 unit/ml as defined by Sigma) in 20 mM sodium acetate buffer, pH 5.0, at a substrate concentration of approx. 3 μM . Wheat bran phytase was used as described [6]. Reactions were stopped by boiling for 5 min and the products neutralized to pH 6–7 with formic acid before HPLC.

RESULTS

InsP₆ was obtained from tissue labelled for 30 min with [^{32}P]P_i, desalted, mixed with [^3H]InsP₆ and subjected to alkaline hydrolysis. The products of alkaline hydrolysis were neutralized and applied to an Adsorbosphere SAX column. The ^{32}P radioactivity of the column eluate was estimated by online Čerenkov counting in a Canberra Packard Radiomatic A500 series flow detector. Fractions (0.5 min) were collected from the detector effluent and either stored for dual-label counting or pooled, desalted and applied to a Partisphere SAX HPLC column. Figure 1 details the profile obtained on Adsorbosphere SAX HPLC. Various peaks of radioactivity were detected and tentatively assigned as InsP₂ species, InsP₃ species, InsP₄ species,

Figure 1 Anion-exchange separation on Adsorbosphere SAX of an alkaline hydrolysate of $[^{32}\text{P}]\text{InsP}_6$

$[^{32}\text{P}]\text{InsP}_6$ was purified by Partisphere SAX HPLC of a perchloric acid extract obtained from 30 min $[^{32}\text{P}]\text{P}_i$ -labelled *Spirodela*. InsP_6 was desalted, mixed with $[^3\text{H}]\text{InsP}_6$ and heated at 120°C for 3 h in 0.5 ml of 50 mM Na_2HPO_4 , pH 10.5 with NaOH. The products were neutralized to pH 6–7 with formic acid, applied to an Adsorbosphere SAX HPLC column and eluted as described in the Materials and methods section. Radioactivity was determined by Čerenkov counting in a Canberra Packard Radiomatic A500 series flow detector with an integration interval of 6 s. Fractions were collected from the detector effluent and retained for dual-label scintillation counting and further analysis. The general positions of elution of the different classes of inositol phosphate are indicated, as are those of the different InsP_5 species whose identity was confirmed by further analysis. The InsP_5 (1/3-OH) peak contains traces of InsP_5 (2-OH); see inset. The inset shows the separation of InsP_5 (1/3-OH) and InsP_5 (2-OH) on a Partisphere SAX HPLC column. A peak of InsP_5 (1/3-OH)/(2-OH) purified by Adsorbosphere SAX HPLC was desalted, applied to a Partisphere SAX HPLC column and eluted as described in the Materials and methods section. Fractions (1 min) were collected and radioactivity was determined by Čerenkov counting. The $^3\text{H}/^{32}\text{P}$ radioactivity of pooled peak fractions was subsequently measured by dual-label scintillation counting.

InsP_5 species and unreacted InsP_6 . Attention was focused on the InsP_5 species, which were identified preliminarily with reference to the published order of elution InsP_5 (5-OH) $\rightarrow$ InsP_5 (4/6-OH) $\rightarrow$ InsP_5 (2-OH)/ InsP_5 (1/3-OH) on Adsorbosphere SAX [11]. Given that the major product of alkaline hydrolysis of InsP_6 is InsP_5 (1/3-OH) [10,15] it is most likely that the major peak of InsP_5 in Figure 1 is InsP_5 (1/3-OH) containing a small amount of InsP_5 (2-OH). This peak was desalted and evidence consistent with the above identification was obtained on subsequent HPLC on Partisphere SAX (inset to Figure 1). This procedure revealed the presence of a major peak of putative InsP_5 (1,2,4,5,6) P_5 (1/3-OH), which was eluted substantially later than a much smaller peak of putative InsP_5 (2-OH).

In other experiments the neutralized alkaline hydrolysate was applied to a Partisphere SAX column. The order of elution of InsP_5 species on this column is InsP_5 (2-OH) $\rightarrow$ InsP_5 (4/6-OH) $\rightarrow$ InsP_5 (5-OH)/ InsP_5 (1/3-OH) [6,10]. In these cases a similar profile of peaks was obtained, with a major peak of putative InsP_5 (1/3-OH) presumably containing traces of InsP_5 (5-OH) being eluted after two minor peaks of putative InsP_5 (2-OH) and InsP_5 (4/6-OH). In this case confirmation that the major peak contained mainly InsP_5 (1/3-OH) was obtained by the demonstration of co-elution of the compound with $[^3\text{H}]\text{InsP}_5$ (1/3-OH) (results not shown). The first-eluted InsP_5 peak was identified as $[^3\text{H}]\text{InsP}_5$ (2-OH) by co-elution with $[^3\text{H}]\text{InsP}_5$ (2-OH) and the second as InsP_5 (4/6-OH) by virtue of the fact that it was eluted

Figure 2 Anion-exchange separation on (a) Adsorbosphere SAX and (b) Partisphere SAX HPLC columns of the products of separate treatments of $[^{32}\text{P}]\text{InsP}_6$ with *Aspergillus* phytase

$[^{32}\text{P}]\text{InsP}_6$ was purified by Partisphere SAX HPLC from perchloric acid extracts of 30 min $[^{32}\text{P}]\text{P}_i$ -labelled *Spirodela*. $[^{32}\text{P}]\text{InsP}_6$ was desalted, mixed with $[^3\text{H}]\text{InsP}_6$ and presented as a substrate to *Aspergillus* phytase in an assay as described in [6]. Reactions were stopped by boiling for 5 min and after neutralization with formic acid the products were applied to (a) Adsorbosphere SAX or (b) Partisphere SAX HPLC columns, both columns being eluted as described in the Materials and methods section. Radioactivity was determined in (a) by Čerenkov counting in a Canberra Packard Radiomatic A500 series flow detector with an integration interval of 6 s (fractions were collected from the detector effluent and retained for dual-label scintillation counting and further analysis) and in (b) by dual-label scintillation counting of fractions (from 1 to 45 min, and 0.5 min thereafter). The general positions of elution of the different classes of inositol phosphate are indicated, as are those of the different InsP_5 species whose identity was confirmed by further analysis (see text). InsP_5 (3-OH) is the major product of treatment of InsP_6 with *Aspergillus* phytase, and InsP_5 (6-OH), with little or no InsP_5 (4-OH), is a minor product [6,12].

on a Partisphere SAX column between $[^3\text{H}]\text{InsP}_5$ (2-OH) and $[^3\text{H}]\text{InsP}_5$ (1/3-OH) (results not shown).

After separation on Adsorbosphere SAX and/or Partisphere SAX HPLC columns, peak fractions corresponding to InsP_5 (2-OH), InsP_5 (4/6-OH), InsP_5 (5-OH), InsP_5 (1/3-OH) and InsP_6 were mixed with scintillant and ^3H and ^{32}P measured by dual-label liquid-scintillation analysis.

Figure 2 shows the HPLC separation on Adsorbosphere SAX (Figure 2a) and Partisphere SAX (Figure 2b) columns of the

Figure 3 Partisphere SAX separation of the InsP_5 (3-OH) product of the treatment of $[^{32}\text{P}]\text{InsP}_6$ with *Aspergillus* phytase

An aliquot of a putative InsP_5 (3-OH) peak purified by Partisphere SAX chromatography of $[^{32}\text{P}]\text{InsP}_6$ treated with *Aspergillus* phytase was mixed with approx. 4500 d.p.m. of acid-hydrolysed $[^3\text{H}]\text{InsP}_5$ (2-OH), re-applied to a Partisphere SAX column and eluted as described in the Materials and methods section. Fractions (0.5 min) were collected and ^3H and ^{32}P radioactivity was determined by dual-label scintillation counting. Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P .

products of separate treatments of $[^{32}\text{P}]\text{InsP}_6$ with *Aspergillus* phytase. Two peaks of InsP_5 were resolved in both cases, a larger peak of InsP_5 (3-OH) and a smaller peak of InsP_5 (6-OH) [6,12]. Confirmation of the identity of the putative InsP_5 (3-OH) peak as InsP_5 (3-OH) was obtained by co-elution of an aliquot (approx. 1000 d.p.m. of ^{32}P) of the peak obtained from the Partisphere SAX-separated material (Figure 2b) with an excess of $[^3\text{H}]\text{InsP}_5$ (1/3-OH), generated by acid-hydrolysis of $[^3\text{H}]\text{InsP}_5$ (2-OH), sufficient to mask the ^3H content (approx. 650 d.p.m.) of the putative InsP_5 (3-OH) peak when rechromatographed on a Partisphere SAX column (Figure 3). This Figure also shows a small peak of what is presumably InsP_5 (4/6-OH), which is eluted between InsP_5 (2-OH) and InsP_5 (1/3-OH) on Partisphere SAX columns, generated by limited transmigration of phosphate on acid hydrolysis.

The results presented in Table 1 show the proportion of the total ^{32}P label in InsP_6 that was lost on removal of a single phosphate to give the InsP_5 species identified. In the experiments in which analysis was restricted to Partisphere SAX columns, resolution of InsP_5 (5-OH) from InsP_5 (1/3-OH) was not achieved. However, InsP_5 (5-OH) is only a minor component of alkaline hydrolysates compared with InsP_5 (1/3-OH) (see Figure 1) and so would be expected not to compromise the $^{32}\text{P}/^3\text{H}$ ratio of the InsP_5 (1/3-OH) peak.

Analysis (Table 1) of the distribution of label in individual phosphates or in racemic mixtures of enantiomers 1/3-OH and 4/6-OH shows that removal of the phosphate in the 2-position released a greater proportion of label from InsP_6 (37% and 32% of the label in duplicate experiments, and 39% in an experiment that employed the *Aspergillus* phytase) than that released by the generation of racemic mixtures of InsP_5 (1/3-OH) or InsP_5 (4/6-OH) (11% and 19% respectively in one experiment, and 13% and 16% respectively in the duplicate experiment). Because alkaline hydrolysis yields racemic mixtures of InsP_5 (1/3-OH) and InsP_5 (4/6-OH), the upper and lower limits for labelling individual phosphates in structurally equivalent positions 1/3 and 4/6 are, for the first experiment (column 1), 22% and 38%

respectively. It is therefore clear that unless we assume that a particular phosphate makes essentially no contribution to the labelling of InsP_6 , and hence that its enantiomeric partner exclusively is labelled, the phosphate in the 2-position is the most strongly labelled of all the phosphates in InsP_6 and must therefore be added last. The results obtained on treatment with *Aspergillus* phytase, which affords considerable (87%) stereospecificity for the 3-position [10] in removal of phosphates from an InsP_6 substrate, demonstrate that phosphates in structurally equivalent positions all show appreciable labelling. Thus the assumption that some of the phosphates do not contribute to the labelling is not upheld and so we conclude that the phosphate in the 2-position is the most strongly labelled and that the addition of the 2-phosphate is the ultimate step in the synthetic sequence to InsP_6 .

Analysis of the labelling of phosphates in the 1- and 3-positions reveals (columns 4 and 5) that where alkaline hydrolysis and phytase treatments were performed in parallel on the same InsP_6 sample, the phosphate in the 1-position (21% of the label) was much more strongly labelled than that in the 3-position (9%). Replicate analyses of the labelling in the 3-position (see columns 6 and 7) gave values of 10% and 10% respectively, confirming that the level of labelling in the 3-position was the lowest of all the phosphates and thus that addition of the phosphate in the 3-position is the first step in the metabolic sequence. It should be pointed out that the calculation of label in the 1-position presented in Table 1 assumes that the enzyme is entirely specific for the 3-position. This is not so. Stephens et al. [10] have reported that the *Aspergillus* phytase is 87% specific for the 3-position of the enantiomerically equivalent phosphates 1 and 3 of InsP_6 . Consequently, the value for the labelling of the 3-position recorded in Table 1 is actually an overestimate of labelling in that position. Similarly, any failure on our part to achieve the selectivity of the *Aspergillus* phytase for an InsP_6 substrate reported [10] would have the consequence that the measured difference in the extent of labelling of the 1- and 3-positions will be increased. That is, the percentage labelling of the 3-position will tend towards zero. Nevertheless it is clear (see Figure 2 in the preceding paper [1]) that in our hands the *Aspergillus* phytase does show considerable, although interestingly in the context of this discussion not absolute, specificity for the 3-phosphate over and above the 1-phosphate, albeit with a monophosphate as substrate.

The metabolic evidence, then, indicates that the 3- and 2-phosphates are the first and last phosphates respectively to be added and this is entirely consistent with the structural evidence presented in [1] that only a single isomer of InsP and one principal InsP_5 , Ins3P and InsP_5 (2-OH) respectively have been identified in *Spirodela* under developmental conditions in which there is massive synthesis and accumulation of InsP_6 .

Similar analyses of the labelling of phosphates in the 4- and 6-positions reveal that the loss of label from InsP_6 on generation of racemic mixtures of 4-OH and 6-OH enantiomers was between 16% and 19%. Very similar values, 16–18%, were obtained on treatment with *Aspergillus* phytase, which yielded InsP_5 (3-OH) predominantly and a minor product that is almost exclusively the 6-OH enantiomer [6]. Significantly, dissection of the same $[^{32}\text{P}]\text{InsP}_6$ sample as that detailed in column 6, but with a commercially available wheat bran phytase specific for the 4-position [6], yielded a value for loss of label of 16% (column 8). The phytases characterized in extracts of wheat bran [13,14] were specific for the 4-position. The striking similarity in the values of loss of label for the 4- and 6-positions under three different dissection regimes and with two different enzymes highly specific for opposite enantiomeric partners provides compelling evidence

Table 1 Analysis of distribution of ^{32}P between the individual phosphate moieties of the InsP_5 products of dissection of $[\text{}^{32}\text{P}]\text{InsP}_6$

$[\text{}^{32}\text{P}]\text{InsP}_6$ was obtained from HClO_4 extracts of $[\text{}^{32}\text{P}]\text{orthophosphate}$ -labelled tissue by HPLC on a Partisphere SAX anion-exchange column. $[\text{}^{32}\text{P}]\text{InsP}_6$ was desalted, mixed with $[\text{}^3\text{H}]\text{InsP}_6$ obtained from $[\text{}^3\text{H}]\text{Ins}$ -labelled tissue, and either hydrolysed in 50 mM Na_2HPO_4 , pH 10.5 with NaOH, or presented as a substrate to *Aspergillus* phytase or wheat bran phytase. The reaction products were neutralized to pH 6–7 and applied either to a Partisphere SAX or Adsorbosphere SAX HPLC column. The ^3H and ^{32}P content of InsP_6 -containing peaks was measured by dual-label scintillation counting. Those InsP_5 peaks containing mixtures of isomers were desalted and applied to a different column, either Partisphere or Adsorbosphere SAX. The ^3H and ^{32}P contents of the peaks resolved on the second column were measured by dual-label scintillation counting. Measurement of the $^3\text{H}/^{32}\text{P}$ ratio of individual peaks of InsP_5 and of the parent InsP_6 allowed the calculation of the proportion of the total label in InsP_6 that was lost on removal of a given phosphate. Each column represents the result of a separate dissection of InsP_6 . The principal product of alkaline hydrolysis of InsP_6 is InsP_5 (1/3-OH) [10,15]. Treatment of InsP_6 with *Aspergillus* phytase yields InsP_5 (3-OH) principally and InsP_5 (6-OH), with little or no InsP_5 (4-OH), as a minor product [6,12]. Treatment of InsP_6 with wheat bran phytase yields InsP_5 (4-OH) principally [6,13,14]. In some experiments the minor products were either not observed or obtained in such low yield as to make measurement of $^3\text{H}/^{32}\text{P}$ ratios in them unreliable. The values in bold type represent the percentage of the ^{32}P label lost from InsP_6 on removal of individual phosphates. The values in parentheses represent the ^3H (upper) and ^{32}P (lower) radioactivity (d.p.m.) of the InsP_5 and InsP_6 fractions from which the values in bold type were calculated. Notes: ^avalue obtained on phytase treatment assumes that isomer obtained is exclusively one enantiomer; ^bvalue obtained for corresponding enantiomer by subtraction of enantiomer ^avalue from that obtained from ^cracemate on alkaline hydrolysis of same InsP_6 sample (see the text); ^dvalue obtained by HPLC separation of monophosphate products; ^evalue obtained by treatment with wheat-bran phytase of an aliquot of the same InsP_6 sample analysed in ^a and ^c; ^fadsorbosphere-SAX-purified peaks obtained on alkaline hydrolysis contain traces of InsP_5 (2-OH); see Figure 1.

Percentage of ^{32}P label in individual phosphates of InsP_6 : dissection of InsP_6								
Position	Alkaline hydrolysis				Phytase treatment			
					<i>Aspergillus</i>		Wheat bran	
2-	37 ^3H (2677) ^{32}P (2011)		32 (5119) (1157)				39^d (88269) (28301)	
					4-OH	14^b		16^e (5214) (4908)
4/6-	19 (7716) (7459)		16 (14934) (4129)	16^c (21090) (7536)				
					6-OH	18^a (20586) (7201)	17 (2825) (4098)	16 (2405) (1652)
					1-OH	21^b		
*1/3-	11 (59664) (63006)	15 (10733) (16325)	13 (88214) (25373)	15^c (58562) (21197)				
					3-OH	9^a (77756) (30189)	10 (5635) (8841)	10 (1666) (1227)
5-			19 (6510) (1740)	23 (9735) (3176)				
InsP_6	(58173) (69093)	(8344) (14874)	(66014) (21788)	(67640) (28775)	(81911) (34870)	(12222) (21255)	(13106) (10731)	(133118) (149921)

that the addition of the 4- and 6-phosphates are neighbouring steps in the metabolic sequence. Although the similarity in the values does not allow us to conclude which of the two phosphates is more strongly labelled and hence is added last, it is clear that the additions of the 4- and 6-phosphates are intermediate to the addition of the 3-phosphate on the one hand and the 1- and 5-phosphates on the other. The structural evidence presented in the accompanying paper [1] is less ambiguous. Two species of InsP_2 were detected in perchloric extracts of *Spirodela*. These were rigorously identified as $\text{Ins}(1,4)\text{P}_2$, presumably derived from lipid turnover, and $\text{Ins}(3,4)\text{P}_2$. Thus, taken together with the identification of $\text{Ins}(3,4,6)\text{P}_3$ as the major InsP_3 species, the only consistent explanation of the results is that the addition of the 4-phosphate precedes that in the 6-position.

A similar line of argument may be followed in consideration of the order of addition of the phosphates in the 1- and 5-positions. Both of these phosphates are more strongly labelled than the 3-, 4- and 6-phosphates but less so than the 2-phosphate. The detection of only a single isomer of InsP_4 and one major isomer of InsP_5 and their identification as $\text{Ins}(3,4,5,6)\text{P}_4$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$ respectively, again under conditions of massive InsP_6 synthesis, is consistent only with the addition of the

phosphate in the 5-position preceding that in the 1-position, that is without invoking the entirely hypothetical generation of $\text{Ins}(1,3,4,6)\text{P}_4$. Also, recalling the overestimation of labelling in the 3-position described above, the values detailed in Table 1 underestimate the labelling of the 1-position in the enantiomerically equivalent pair. The consequence of this is that the labelling of the 1-phosphate will be greater than that of the 5-phosphate, further supporting the circumstantial and most parsimonious explanation of the evidence that the one detectable InsP_4 isomer, $\text{Ins}(3,4,5,6)\text{P}_4$, is the metabolic precursor of $\text{Ins}(1,3,4,5,6)\text{P}_5$.

Thus from the foregoing we conclude that in *Spirodela polyrhiza* the metabolic route by which InsP_6 is synthesized from inositol proceeds according to the sequence $\text{Ins}3\text{P} \rightarrow \text{Ins}(3,4)\text{P}_2 \rightarrow \text{Ins}(3,4,6)\text{P}_3 \rightarrow \text{Ins}(3,4,5,6)\text{P}_4 \rightarrow \text{Ins}(1,3,4,5,6)\text{P}_5 \rightarrow \text{InsP}_6$.

DISCUSSION

The results presented above and in the preceding paper [1] establish for the first time in the plant kingdom, and only for the second time in any kingdom, the route of synthesis of InsP_6 ,

specifically in the aquatic monocotyledonous plant *Spirodela polyrhiza* L. It is therefore remarkable that the pathway described differs from that established [2] for the slime mould *Dictyostelium discoideum*, but in common with that organism shows no obvious direct link to second messenger function. The pathway described above $[\text{Ins}3P \rightarrow \text{Ins}(3,4)P_2 \rightarrow \text{Ins}(3,4,6)P_3 \rightarrow \text{Ins}(3,4,5,6)P_4 \rightarrow \text{Ins}(1,3,4,5,6)P_5 \rightarrow \text{Ins}P_6]$ shares three intermediates, $\text{Ins}3P$, $\text{Ins}(3,4,6)P_3$ and $\text{Ins}(1,3,4,5,6)P_5$, with a pathway $[\text{Ins}3P \rightarrow \text{Ins}(3,6)P_2 \rightarrow \text{Ins}(3,4,6)P_3 \rightarrow \text{Ins}(1,3,4,6)P_4 \rightarrow \text{Ins}(1,3,4,5,6)P_5 \rightarrow \text{Ins}P_6]$ described for *Dictyostelium*. It may therefore be significant that despite the widespread phylogenetic occurrence of $\text{Ins}(3,4,6)P_3$ in avian erythrocytes [3,16], WRK1 rat mammary tumour cells [17], the slime mould *Dictyostelium* [2] and the plant *Spirodela* (see preceding paper [1]), and similarly although also more widely [10] of $\text{Ins}(1,3,4,5,6)P_5$, the strongest evidence seems to indicate functions for both these isomers as intermediates in the pathway of $\text{Ins}P_6$ synthesis.

More significantly, there is strong evidence that the partial sequence $\text{Ins}(3,4,6)P_3 \rightarrow \text{Ins}(3,4,5,6)P_4 \rightarrow \text{Ins}(1,3,4,5,6)P_5$ is common to plant and animal kingdoms. $\text{Ins}(3,4,5,6)P_4$ has been detected in many cell types including avian erythrocytes and bone macrophages [18], B-lymphocytes [19], WRK1 rat mammary tumour cells [17] and bovine adrenal glomerulosa cells [20]. $\text{Ins}(3,4,5,6)P_4$ serves as a precursor of $\text{Ins}(1,3,4,5,6)P_5$ in homogenates of bone macrophages, of various rat tissues [21] and in bovine adrenal glomerulosa cells [20]. More directly, the metabolic data presented [3] indicate that $\text{Ins}(3,4,5,6)P_4$ is derived from $\text{Ins}(3,4,6)P_3$ and is itself the major precursor of $\text{Ins}(1,3,4,5,6)P_5$ in avian erythrocytes. Thus from the foregoing the entire partial sequence $\text{Ins}(3,4,6)P_3 \rightarrow \text{Ins}(3,4,5,6)P_4 \rightarrow \text{Ins}(1,3,4,5,6)P_5$ seems common to avian and plant cells and perhaps also to animal cells. It is thus tempting to speculate that the last three steps in the biosynthesis of $\text{Ins}P_6$ are common to avian, mammalian and plant cells.

An alternative role for $\text{Ins}(3,4,5,6)P_4$ and $\text{Ins}(1,3,4,5,6)P_5$ is implied by evidence that links $\text{Ins}(3,4,5,6)P_4$, somewhat indirectly, to receptor activation. Levels of $\text{Ins}(3,4,5,6)P_4$, but also of $\text{Ins}(1,4,5,6)P_4$ and $\text{Ins}(3,4,5,6)P_4$, are elevated in angiotensin-stimulated bovine adrenal glomerulosa cells [20], in vasopressin-stimulated WRK1 rat mammary tumour cells [22] and in bombesin-stimulated rat pancreatoma cells [23,24]. Whereas in the latter case it appears that $\text{Ins}(3,4,5,6)P_4$ is the product of an $\text{Ins}(1,3,4,5,6)P_5$ 1-phosphatase activity and that $\text{Ins}(1,3,4,6)P_4$ is the *de novo* precursor of $\text{Ins}(1,3,4,5,6)P_5$, synthesis of $\text{Ins}(1,3,4,5,6)P_5$ can proceed from $\text{Ins}(1,4,5)P_3$ in homogenates of rat brain [25] and hence may be linked to receptor activation. In contrast $\text{Ins}(3,4,5,6)P_4$ production does not seem to be linked to phosphoinositide hydrolysis in WRK1 rat mammary tumour cells [22]; rather the source of $\text{Ins}(3,4,5,6)P_4$ in this tissue remains undefined.

Notwithstanding the above, the evidence presented in the present study and for avian erythrocytes [3], that the 3-phosphate is added first and the 1-phosphate much later in the sequence clearly, discounts any link between the inositol phosphate intermediates of the sequence to $\text{Ins}P_6$ in *Spirodela* and those of the partial sequence $\text{Ins}(3,4,6)P_3 \rightarrow \text{Ins}(3,4,5,6)P_4 \rightarrow \text{Ins}(1,3,4,5,6)P_5$ in avian erythrocytes, from those associated with receptor-activated phosphoinositide hydrolysis in which the 1-phosphate is added first.

The identification of $\text{Ins}3P$ in *Spirodela* is not in itself unsurprising, given the pioneering work of Loewus and co-workers on inositol biosynthesis and metabolism (reviewed in [26]). Thus 1-L-*myo*-inositol-1-phosphate synthase, which catalyses the cyclization of glucose 6-phosphate to yield $\text{Ins}3P$, has a unique role in the provision of $\text{Ins}3P$ for the synthesis of *myo*-

inositol *de novo*. Alternatively it has been shown that $\text{Ins}3P$ is also the product of *myo*-inositol kinase. The enantiomeric nature of the product was first determined [27] in a study that, along with the work of Lim and Tate [14] and others, established a precedent for the rigorous identification of inositol phosphates in plants but which has rarely been matched since. The fact that in the present study $\text{Ins}3P$ is derived from exogenous *myo*-inositol clearly identifies *myo*-inositol kinase in *Spirodela*. Moreover, the additional evidence that inositol moieties destined for $\text{Ins}P_6$ synthesis are initially phosphorylated in the 3-position establishes a role for *myo*-inositol kinase as the first step in the pathway to $\text{Ins}P_6$ from inositol in *Spirodela*, as in *Dictyostelium* [2,28]. The experiments described above do not allow us to determine the direct contribution to $\text{Ins}P_6$ synthesis of 1-L-*myo*-inositol-1-phosphate synthase.

A caveat to the metabolic analysis described above is that the data are not compromised by substrate-cycles. It is worth addressing the possibility, however hypothetical, that the validity of the metabolic analysis may be compromised by substrate cycling of phosphate ester groups between different inositol phosphates. That such substrate cycles may exist has been suggested [2] wherein it was shown that the three isomers of $\text{Ins}P_5$ detected in *Dictyostelium* were all substrates for kinase(s) producing $\text{Ins}P_6$ *in vitro*. One, which was the sole product of an $\text{Ins}P_4$ kinase *in vitro*, turned over more slowly both *in vitro* and *in vivo* than the other two, which seemed to participate in substrate-cycles with $\text{Ins}P_6$. It remains to be established whether this turns out to be a universal phenomenon. Other authors [29] have speculated on the basis of the range of inositol phosphate kinase and phosphatase activities that can be detected in animal cell homogenates on the possibility of substrate-cycles involving other inositol phosphates. That such substrate-cycles exist is far from established and must await the sort of analysis described above [2] or alternatively the sort of metabolic description borne out of labelling *in vivo* and analysis of the labelling of individual phosphate esters described in earlier work on inositol lipid metabolism [8]. In the context of the present study the potentially compromising possibility of substrate-cycles must be tempered by the available evidence.

First, any hypothetical substrate-cycle can involve only those isomers detected. Thus the existence of a single $\text{Ins}P_4$ isomer does not allow for substrate-cycles involving other than $\text{Ins}P_5$ and $\text{Ins}P_6$. This aside, there is no direct evidence that $\text{Ins}P_4$ species or lower phosphates of inositol are components of substrate-cycles *in vivo*, in which case the only potentially compromising situation is that raised by the presence of a trace, less than 1% of total $\text{Ins}P_5$ species, of the 1/3-OH isomer and its hypothetical involvement in a substrate-cycle. Set against the massive accumulation of $\text{Ins}P_6$ shown in Figure 1 of the preceding paper [1] and the analysis described above, which indicates that the phosphate in the 2-position is the last to be added to the inositol moiety to make $\text{Ins}P_6$, the most parsimonious explanation is that hypothetical substrate-cycles do not compromise our data. Because it is not beyond doubt that the phosphate in the 1-position is more strongly labelled than that in the 5-position it is feasible, although not supported by the analysis of inositol phosphates present in *Spirodela* in the preceding paper [1], that this part of the sequence is the same as that described in *Dictyostelium* [2].

Returning to the question of substrate-cycles, we should also be aware of the existing cautions in the literature [30] against the universal application of animal signalling paradigms to plants. In particular the accumulation of $\text{Ins}P_6$ to levels in plants far in excess of those described in animals seems to represent a fundamental difference between the plant and animal kingdoms.

Therefore the fact that the range of inositol phosphate isomers detected in *Spirodela polyrhiza* [1] is completely atypical of that described in the animal kingdom and seems to have little to do with phospholipid-mediated signalling suggests that most of the isomers detected are associated with the considerable synthesis of InsP_6 occurring in this tissue. Furthermore the isomers detected in *Spirodela polyrhiza* are not those described [15] as the products of breakdown of InsP_6 by phytases of plant, microbiological or fungal origin.

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