Inositol phosphates in barley (*Hordeum vulgare* L.) aleurone tissue are stereochemically similar to the products of breakdown of InsP_6 *in vitro* by wheat-bran phytase

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Partisphere SAX HPLC analysis of endogenous inositol phosphates in [^3H]inositol-labelled barley aleurone tissue revealed a range of isomers, including D- and/or L- $\text{Ins}3\text{P}$, D- and/or L- $\text{Ins}(1,4)\text{P}_2$, D- and/or L- $\text{Ins}(1,2)\text{P}_2$, a third unidentified InsP_2 , $\text{Ins}(1,2,3)\text{P}_3$, D- and/or L- $\text{Ins}(1,2,6)\text{P}_3$, D- and/or L- $\text{Ins}(1,2,3,4)\text{P}_4$, D- and/or L- $\text{Ins}(1,2,5,6)\text{P}_4$, $\text{Ins}(1,3,4,5,6)\text{P}_5$, D- and/or L- $\text{Ins}(1,2,3,4,5)\text{P}_5$, $\text{Ins}(1,2,3,4,6)\text{P}_5$, InsP_6 and a molecule with the chromatographic properties of an inositol pyrophosphate. The

striking match between the identities of the stereoisomers, and in some cases enantiomers, detected *in vivo* and those stereoisomers produced *in vitro* by the action of wheat-bran phytase on InsP_6 [Cosgrove (1980) Inositol Phosphates: Their Chemistry, Biochemistry and Physiology, Elsevier, Amsterdam] strongly suggests that most of the inositol phosphates identified are products of the breakdown of InsP_6 by endogenous phytase(s) with stereospecificity similar to that of the wheat-bran enzyme(s).

INTRODUCTION

Early studies of phytic acid (InsP_6) synthesis in a number of plant tissues [1–7] reported the presence on anion-exchange chromatography of a range of peaks labelled from [^3H]inositol, [^{14}C]inositol and [^{32}P]P_i. Although some of these peaks, and particularly the more polar ones, are likely to be inositol phosphates, the diverse metabolic fates of both *myo*-inositol and P_i in plant tissues have the consequence that many of the peaks which are eluted from anion-exchange columns are unlikely to be inositol phosphates. Moreover, despite considerable interest in inositol phospholipid-mediated signal transduction pathways in plants, virtually nothing is known of the stereochemical identities of inositol phosphates in plants. We have recently identified a number of inositol phosphates in *Spirodela polyrhiza* which, from a metabolic analysis of the order of addition of phosphates to inositol, appear to be intermediates in the metabolic sequence from inositol to InsP_6 [8,9]. It is not possible, however, to say whether the peaks of putative inositol phosphates detected in plants in earlier studies are intermediates in pathways of InsP_6 synthesis or breakdown, or whether they occur in pathways of metabolism of signalling molecules in plants.

In a notable departure from the uncertainty that surrounds plant inositol phosphate metabolism, Loewus and co-workers in their pioneering studies of sugar metabolism, reviewed in [10], were able to establish by chiral gas chromatography the enantiomerism of the InsP product of the enzymic cyclo-aldolization of glucose 6-phosphate. They were subsequently able to show [11] that the $\text{Ins}3\text{P}$ product of cyclo-aldolization is identical to the product, enantiomerically uncharacterized, of the ATP-dependent *myo*-inositol kinase activities previously found in plants, animals and micro-organisms.

D- and/or L- $\text{Ins}(1,2,3,4)\text{P}_4$, $\text{Ins}(1,2,5,6)\text{P}_4$, D- and L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ and D- and/or L- $\text{Ins}(1,2,3,4,5)\text{P}_5$ have been

identified in germinating mung bean seedlings [12,13], while all of the peaks of InsP_5 which are resolvable on non-chiral HPLC systems, i.e. $\text{Ins}(1,3,4,5,6)\text{P}_5$, D- and/or L- $\text{Ins}(1,2,3,4,5)\text{P}_5$, $\text{Ins}(1,2,3,4,6)\text{P}_5$ and D- and/or L- $\text{Ins}(1,2,4,5,6)\text{P}_5$, were identified in soybean [14]. In addition, we have described the range of inositol phosphates present in the duckweed *Spirodela polyrhiza* L. [8]. Unlike many of the plant systems studied to date, barley aleurone tissue represents a single cell type which is not only responsive to a wide range of hormonal stimuli but which also, during the course of seed maturation, accumulates large amounts of phytic acid in the globoids of aleurone grains. During germination, phytic acid is mobilized by the action of phytases, the best characterized of which, in terms of *in vitro* substrate specificity, are probably the phytases of wheat-bran (reviewed in [15]). The range of inositol phosphate isomers produced *in vivo* on mobilization of phytic acid remains to be established. Because of the considerable historical interest in the aleurone tissue as a model system of plant–hormone interactions, and one with many implications for inositol phosphate and particularly InsP_6 metabolism, we have chosen in the present study to characterize the inositol phosphate content of barley aleurone tissue.

MATERIALS AND METHODS

Isolation and labelling of aleurone tissue

Seeds of barley (*Hordeum vulgare* L. cv Himalaya, 1993 harvest), obtained from the University Botanic Gardens, Cambridge, U.K., were de-embryonated and surface sterilized in bleach (2.5 % available chlorine) for 40 min. The de-embryonated grains were washed five times in sterile water and allowed to imbibe for 3 days under aseptic conditions on filter paper in 90 mm Petri dishes containing 5 ml of 10 mM L-arginine, 20 mM CaCl_2 and 20 $\mu\text{g/ml}$ chloramphenicol.

Abbreviation used: Gro $\text{Ins}3\text{P}$, glycerophosphoinositol 3-phosphate. Nomenclature: inositol phosphates from biological sources in which the ratio of enantiomers is undefined are given the prefix D- and/or L-; all other isomers, except *meso*-compounds, are assigned as D-isomers.

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Aleurone layers from 35 grains were manually isolated from the starchy endosperm under aseptic conditions. Isolated aleurone layers were incubated at 25 °C in the dark for 48 h in 2.1 ml of 50 mM succinate buffer (pH 5.0 with NaOH, 25 °C), 10 mM CaCl₂ and 20 µg/ml chloramphenicol, containing 100 µCi of *myo*-[2-³H]inositol (Amersham).

Tissue extraction

After labelling, aleurone layers were washed with water, blotted dry, weighed and frozen in liquid N₂. At this stage an aliquot (50 µl) of phytate hydrolysate (40 µg of phosphorus) was added to minimize losses of inositol phosphates due to non-specific binding during extraction [8]. The tissue was ground in a liquid nitrogen-cooled mortar and pestle and extracted with 0.8 ml of 3.5 % (w/v) HClO₄. The cell debris was pelleted by centrifugation (13000 g, 5 min) in a refrigerated Microfuge and re-extracted with 0.3 ml of HClO₄. The pellet was retained for immediate extraction of lipids. The supernatant was neutralized to pH 6–7 with 2 M KOH, 100 mM Mes and 10 mM EDTA, and held on ice for 15 min to precipitate KClO₄ before centrifugation (3000 g, 5 min) in a refrigerated bench centrifuge. The resulting supernatant was stored at –20 °C before HPLC.

HPLC

Perchloric acid extracts of [³H]inositol-labelled tissue were resolved on Partisphere SAX and Adsorbosphere SAX HPLC columns. The gradients used for separation of different classes of inositol phosphates, and for different stereoisomers among the different classes, have been described [8,9]. Inositol phosphate fractions were desalted on Dowex AG1 X8 (formate form) resin and, where appropriate, subjected to mild acid-catalysed phosphate migration.

Periodate oxidation, reduction and dephosphorylation

HPLC-purified and desalted inositol phosphate fractions were subjected to periodate oxidation, reduction and dephosphorylation. The polyol products were resolved on Polypore Pb²⁺ and aminopropyl HPLC columns [8,16].

Reagents

myo-[2-³H]Inositol, *D*-*myo*-[U-¹⁴C]Ins3P and [³²P]P_i (carrier-free) were obtained from Amersham International (Amersham, Bucks., U.K.).

Standards

[¹⁴C]Ins2P and *D*/L-[¹⁴C]Ins4P were prepared by acid-catalysed phosphate migration (1 M HCl; 100 °C for 10 min) of [¹⁴C]Ins3P. [³²P]Ins(1,3)P₂ and *D*/L-[³²P]Ins(1,2)P₂ were prepared by acid treatment of [³²P]glycerophosphoinositol 3-phosphate ([³²P]GroPIns3P), which was obtained from [³²P]P_i-labelled *Spirodela polyrrhiza* [16]. [³²P]Ins(1,4)P₂, [³²P]Ins(1,5)P₂ and [³²P]Ins(4,5)P₂ were prepared from inositol lipids obtained from [³²P]P_i-labelled turkey erythrocytes [12]. Lipid extracts were deacylated with monomethylamine and deglycerated to yield a mixture of inositol mono-, bis- and tris-phosphates [16]. [³²P]Ins(1,4)P₂, [³²P]Ins(1,5)P₂ and [³²P]Ins(4,5)P₂ were subsequently obtained from inositol phosphates by acid treatment or alkaline hydrolysis [17]. Alternatively, dried lipid extracts were treated directly with acid or alkali.

[³²P]Ins(1,4,5)P₃ was obtained from lipid extracts of turkey erythrocytes. *D*/L-[³²P]Ins(1,2,3,4)P₄ and [³²P]Ins(1,2,5,6)P₄ were

obtained from [³²P]P_i-labelled germinating mung beans according to [12].

[³²P]Ins(1,3,4,5,6)P₅ was obtained from [³²P]P_i-labelled turkey erythrocytes [12]. *D*/L-[³²P]Ins(1,2,4,5,6)P₅ and traces of *D*/L-[³²P]Ins(1,2,3,5,6)P₅ were obtained from [³²P]Ins(1,3,4,5,6)P₅ by acid-catalysed phosphate migration.

RESULTS

Anion-exchange chromatography of the products of labelling of aleurone layers from [³H]inositol on Partisphere SAX HPLC columns revealed a range of peaks of what was assumed to represent an increasing degree of phosphorylation (Figure 1). The most strongly labelled component (other than inositol), which in various experiments accounted for up to approx. 60 % of the label recovered, eluted with a retention time typical of InsP₆. The numerous peaks of material, assumed to be intermediate in degree of phosphorylation, between inositol and InsP₆ were much less strongly labelled. Such a pattern of labelling is typical of that described in some early studies of InsP₆ metabolism. The analysis described below was designed to determine the stereochemical identities of the various peaks.

InsPs

Partisphere SAX HPLC of HClO₄ extracts from [³H]inositol-labelled aleurone tissue revealed a single peak of material with chromatographic properties typical of InsPs, i.e. a peak which co-eluted with an authentic standard of [¹⁴C]Ins3P. This peak was desalted, spiked with a mixture of *D*/L-[¹⁴C]Ins3P, [¹⁴C]Ins2P and traces of [¹⁴C]Ins4P prepared by acid-catalysed phosphate migration of [¹⁴C]Ins3P, and re-applied to the same column. The column was eluted isocratically with 40 mM NaH₂PO₄. Under these more stringent conditions a single ³H-labelled peak was

Figure 1 Inositol phosphates in barley (*Hordeum vulgare*) aleurone tissue

Aleurone layers were isolated from de-embryonated and imbibed barley grains. Layers were labelled for 48 h with *myo*-[³H]inositol. A perchloric acid extract was prepared, neutralized and an aliquot applied to a Partisphere SAX column eluted with a gradient of NaH₂PO₄. All steps were as described in the Materials and methods section. The ³H content of the column eluate was determined by on-line 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 12 s. The approximate positions of elution of the different classes of inositol phosphate identified in aleurone tissue are indicated.

Figure 2 InsPs in aleurone layers

A peak of [^3H]inositol-labelled material which eluted on Partisphere SAX columns in the approximate position of elution of authentic InsPs was desalted, mixed with an aliquot of acid-treated D-[^{14}C]Ins3P, applied to the same column and eluted with 40 mM NaH_2PO_4 . The ^3H and ^{14}C content of the column fractions was determined by on-line 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 in a ratio of 1:2 (v/v). Scintillation events were counted with an integration interval of 12 s. $\circ$, ^3H ; $\bullet$, ^{14}C .

detected which co-eluted precisely with D/L-[^{14}C]Ins3P, and before [^{14}C]Ins2P and [^{14}C]Ins4P (Figure 2). As Ins5P elutes after Ins2P from this column [18], it is clear that the unknown ^3H -labelled peak could contain, of the six stereoisomers of InsP, only D- and/or L-Ins3P. No attempt was made to determine the enantiomerism of this material.

Ins P_2 s

A peak of ^3H -labelled material which in some experiments was resolved into two components, and which eluted with a retention time typical of Ins P_2 s, was detected when HClO_4 extracts were resolved on Partisphere SAX columns (Figure 1). This peak was resolved into two components when HClO_4 extracts or the peak fractions of material from the above gradient were desalted and eluted isocratically from the same column with 212 mM NaH_2PO_4 . Under these conditions the first-eluting and major [^3H]Ins P_2 eluted after a standard of [^{32}P]Ins(1,3) P_2 and before D/L-[^{32}P]Ins(1,2) P_2 , both prepared by acid treatment of [^{32}P]GroPIns3P obtained from *Spirodela polyrhiza* [16]. The second peak of ^3H co-eluted precisely with D/L-[^{32}P]Ins(1,2) P_2 (Figure 3, top panel). In a separate run the first ^3H peak co-eluted precisely with [^{32}P]Ins(1,4) P_2 (results not shown). In a similar run [^3H]Ins(1,3) P_2 and D/L-[^3H]Ins(1,2) P_2 , prepared from [^3H]GroPIns3P, were mixed with [^{32}P]Ins(1,5) P_2 and [^{32}P]Ins(2,5) P_2 , and all four components were resolved (Figure 3, middle panel). [^{32}P]Ins(2,5) P_2 was prepared by acid-catalysed phosphate migration of [^{32}P]Ins(1,5) P_2 , itself prepared by alkaline hydrolysis of [^{32}P]Ins(1,4,5) P_3 , obtained as described below. The Ins P_2 products were separated in order of increasing retention time: Ins(1,4) P_2 , Ins(1,5) P_2 and Ins(4,5) P_2 . It is clear from the foregoing that the chromatographic properties of Ins(1,2) P_2 , Ins(1,5) P_2 and Ins(2,5) P_2 , and perhaps Ins(1,4) P_2 , are very similar. Nevertheless, chromatography of a desalted InsP and Ins P_2 fraction from a ^3H -labelled aleurone extract with [^{32}P]Ins(1,5) P_2 and [^{32}P]Ins(2,5) P_2 resolved four component Ins P_2 s, with the second peak of ^3H -labelled material eluting between Ins(1,5) P_2 and Ins(2,5) P_2 and the first ^3H peak eluting before Ins(1,5) P_2 (Figure 3, bottom panel).

Figure 3 Identification of Ins P_2 s in aleurone layers

Perchloric acid extracts were obtained from [^3H]Ins-labelled aleurone layers and mixed with ^{32}P -labelled standards, and component Ins P_2 s were resolved by anion-exchange chromatography on Partisphere SAX. Columns were eluted with 212 mM NaH_2PO_4 . Top: separation of aleurone [^3H]Ins P_2 s from [^{32}P]Ins(1,3) P_2 and D/L-[^{32}P]Ins(1,2) P_2 . ^{32}P -labelled standards were obtained by acid treatment of [^{32}P]GroPIns3P obtained from *Spirodela polyrhiza* [16]. Middle: separation of [^3H]Ins(1,3) P_2 and D/L-[^3H]Ins(1,2) P_2 from [^{32}P]Ins(1,5) P_2 and D/L-[^{32}P]Ins(2,5) P_2 . ^3H -labelled standards were prepared by acid treatment of [^3H]GroPIns3P. [^{32}P]Ins(1,5) P_2 was prepared by alkaline hydrolysis of [^{32}P]Ins(1,4,5) P_3 , itself obtained from turkey erythrocytes. [^{32}P]Ins(2,5) P_2 was prepared by acid treatment of [^{32}P]Ins(1,5) P_2 . In all cases, radioactivity was determined by on-line scintillation counting with a Canberra Packard Radiomatic A500 series flow detector fitted with a 2 ml flow cell. The column eluate was mixed with Canberra Packard Flo Scint IV scintillation cocktail in a ratio of 1:2 (v/v). Scintillation events were counted with an integration interval of 6 s. $\circ$, ^3H ; $\bullet$, ^{32}P .

To summarize, the first peak of ^3H -labelled material co-eluted precisely with Ins(1,4) P_2 , after Ins(1,3) P_2 and before Ins(1,5) P_2 , Ins(2,5) P_2 and Ins(1,2) P_2 . The second peak of ^3H -labelled material co-eluted precisely with Ins(1,2) P_2 , after Ins(1,3) P_2 , Ins(1,4) P_2 and Ins(1,5) P_2 , but before Ins(2,5) P_2 . In a single separate run under identical chromatographic conditions we were able to resolve, in order of increasing retention time, peaks of Ins(1,3) P_2 , Ins(1,4) P_2 , Ins(1,2) P_2 , Ins(3,4) P_2 and Ins(4,5) P_2 (results not shown). Such a separation on Partisphere SAX has already been described [19], and the relatively late-eluting nature of Ins(3,4) P_2 and Ins(4,5) P_2 is well documented. We have also

Figure 4 Polypore Pb^{2+} and aminopropyl HPLC separations of the polyol products of periodate oxidation, reduction and dephosphorylation of an aleurone InsP_3

Upper panel: an $[\text{3H}]\text{InsP}_3$ fraction, the earliest-eluting of those detected on Partisphere SAX HPLC of $[\text{3H}]\text{inositol}$ -labelled aleurone tissue, was desalted and subjected to periodate oxidation, reduction and dephosphorylation to yield $[\text{3H}]\text{polyols}$. The products were mixed with an authentic standard of $[\text{14C}]\text{ribitol}$ and resolved on a Brownlee Polypore Pb^{2+} column eluted with water at a flow rate of 0.2 ml/min. The injection volume was 50 μl . Fractions (0.5 min) were collected and the ^3H and ^{14}C contents determined by dual-label scintillation counting. The positions of elution of unlabelled polyol standards were determined in a parallel HPLC run by measurement of UV absorbance at 190 nm (all steps as described in the Materials and methods section). $\circ$, ^3H ; $\bullet$, ^{14}C ; solid line, UV absorbance in arbitrary units. Lower panel: the products of periodate oxidation, reduction and dephosphorylation were mixed with authentic standards of $[\text{14C}]\text{ribitol}$ and $[\text{14C}]\text{inositol}$, reduced to dryness and resolved on an aminopropyl HPLC column eluted with acetonitrile/water at a flow rate of 1 ml/min according to [16]. Fractions (0.5 min) were collected and the ^3H and ^{14}C contents determined by dual-label scintillation counting. The positions of elution of unlabelled polyol standards indicated were determined in the same HPLC run by measurement of UV absorbance at 190 nm (all steps as described in the Materials and methods section). $\circ$, ^3H ; $\bullet$, ^{14}C .

described evidence that the product of *Aspergillus* (D-3) phytase attack on $\text{Ins}(3,4,6)\text{P}_3$ yields an InsP_2 product, presumably $\text{Ins}(4,6)\text{P}_2$, which co-elutes with $\text{Ins}(4,5)\text{P}_2$ [8]. The upshot of all this experimental evidence is that if the ^3H -labelled compound in the first peak is an inositol phosphate it can only be $\text{Ins}(1,4)\text{P}_2$ and/or its enantiomer $\text{Ins}(3,6)\text{P}_2$; $\text{Ins}(2,4)\text{P}_2$ elutes after $\text{Ins}(1,4)\text{P}_2$ on this column [17]. We were also able to show that both ^3H -labelled peaks eluted before $\text{Ins}(3,4)\text{P}_2$ (results not shown). Similarly, our ability to distinguish the second eluting ^3H -labelled peak from both of the peaks of the products of the acid-catalysed phosphate migration of $\text{Ins}(1,4)\text{P}_2$ (results not shown), together with all the above evidence, indicates that, if it is an inositol phosphate, the identity of the second ^3H -labelled peak is $\text{Ins}(1,2)\text{P}_2$ and/or its enantiomer $\text{Ins}(2,3)\text{P}_2$.

The uncertainty as to whether the peaks are inositol phosphates or not arises from consideration of the diverse metabolic fates of *myo*-inositol in plant tissues. The most compromising possibility is that these peaks may be intermediates in the synthesis of cell

wall components [5,10,20], although the functionally and terminally differentiated nature of the mature aleurone cell in imbibed endosperms, an experimental system in which there is net cell wall breakdown, means that these peaks are less likely to be intermediates of cell wall synthesis than would be the case for a dividing cell in which net cell wall synthesis is occurring.

In consideration of the above, we tested the possibility that these peaks may not be inositol phosphates in the following manner. Inositol bisphosphates were purified by HPLC on a Partisphere SAX column eluted with 210 mM NaH_2PO_4 . Aliquots of HPLC fractions were taken for estimation of ^3H content by scintillation counting. In addition to the two putative peaks of InsP_2 s identified above, a third late-eluting and minor peak of ^3H -labelled material was detected. This peak was not characterized further, other than to show (see the following) that the ^3H label was associated with inositol. The three peaks of putative InsP_2 s were desalted on Dowex AG-1X8 resin (formate form) and incubated in an assay containing 100 mM ethanalamine/HCl buffer, pH 10, 10 mM MgCl_2 , 1 mM ZnCl_2 and 250 units of alkaline phosphatase (Boehringer 567744) at 25 °C overnight. The reaction products were applied to a column (2 ml bed volume) of mixed-bed ion-exchange resin, and the neutral components were eluted after 30 min with water to give a final volume of 7 ml and freeze dried. The products were mixed with approx. 4600 d.p.m. of *myo*- $[\text{14C}]\text{inositol}$ (Amersham) in acetonitrile/water (85:15, v/v) and applied to an aminopropyl HPLC column as described in [8,16]. All three peaks of putative InsP_2 s yielded a single uncharged product which co-eluted precisely with authentic $[\text{14C}]\text{inositol}$ on this HPLC system (results not shown). We therefore conclude that the parent compounds are the inositol bisphosphates tentatively identified above, namely $\text{Ins}(1,4)\text{P}_2$ and/or $\text{Ins}(3,6)\text{P}_2$, and $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_2$, while the third peak is an unidentified inositol phosphate.

InsP_3 s

Vigorous periodate oxidation, reduction and dephosphorylation of the principal ^3H -labelled InsP_3 peak by the method of [19,21] and as described in [8] generated a product which, when applied to a Polypore Pb^{2+} HPLC column, co-eluted precisely with an authentic standard of $[\text{14C}]\text{ribitol}$, and before unlabelled standards of arabitol, altritol, xylitol, glucitol and iditol (Figure 4, upper panel). The production of ribitol identifies the parent InsP_3 as $\text{Ins}(1,2,3)\text{P}_3$. Such a result is, however, potentially compromised by the difficulty in obtaining separation of inositol and ribitol on such columns; inositol is obtained from both the *meso* compounds $\text{Ins}(1,3,5)\text{P}_3$ and $\text{Ins}(2,4,6)\text{P}_3$. Therefore, in order to provide an unambiguous identification of the polyol product as ribitol, the product of oxidation, reduction and dephosphorylation was applied to an amino HPLC column and eluted as described [16] under conditions which resolved inositol and ribitol. The product co-eluted precisely with a standard of $[\text{14C}]\text{ribitol}$ and not with inositol (Figure 4, lower panel). This result unambiguously identifies the parent inositol phosphate as $\text{Ins}(1,2,3)\text{P}_3$.

The identity of the second, late-eluting, peak of ^3H -labelled InsP_3 material (retention time approx. 31.5 min in Figure 1) was also pursued by periodate oxidation, reduction, dephosphorylation and HPLC. The major peak of the polyol products eluted in a position characteristic of arabitol, i.e. after inositol but before altritol and ^{14}C -labelled standards of xylitol and glucitol (Figure 5). The generation of arabitol on periodate oxidation, reduction and dephosphorylation identifies the parent inositol phosphate as D- and/or L- $\text{Ins}(1,2,6)\text{P}_3$.

Figure 5 Polypore Pb^{2+} HPLC separation of the polyol products of periodate oxidation, reduction and dephosphorylation of a second aleurone InsP_3

A second ^3H InsP_3 fraction obtained from ^3H inositol-labelled aleurone tissue was desalted and subjected to periodate oxidation, reduction and dephosphorylation to yield ^3H polyols. The products were mixed with authentic standards of ^{14}C inositol, ^{14}C xylitol and D - ^{14}C glucitol, and resolved on a Brownlee Polypore Pb^{2+} column eluted with water at a flow rate of 0.2 ml/min. The injection volume was 50 μl . Fractions (0.5 min) were collected and the ^3H and ^{14}C contents determined by dual-label scintillation counting. The positions of elution of unlabelled polyol standards were determined in a parallel HPLC run by measurement of UV absorbance at 190 nm (all steps as described in the Materials and methods section). $\circ$, ^3H ; $\bullet$, ^{14}C ; solid line, UV absorbance in arbitrary units.

Figure 6 Identification of InsP_4 s in aleurone layers

A desalted ^3H InsP_4 fraction, the earlier-eluting from a Partisphere SAX HPLC column of two detected in ^3H Ins -labelled aleurone layers, was mixed with D - and/or L - ^{32}P $\text{Ins}(1,2,3,4)\text{P}_4$ and ^{32}P $\text{Ins}(1,2,5,6)\text{P}_4$, obtained from germinating mung beans according to [12]. The sample was applied to a Partisphere SAX column and eluted with a gradient optimized for separation of InsP_4 s [12]. Radioactivity was determined by on-line scintillation counting with a Canberra Packard Radiomatic A500 series flow detector fitted with a 2 ml flow cell. The column eluate was mixed with Canberra Packard Flo Scint IV scintillation cocktail at a ratio of 1:4 (v/v). Scintillation events were counted with an integration interval of 6 s. $\circ$, ^3H ; $\bullet$, ^{32}P . The data were five-point smoothed using the flow-detector software.

InsP_4 s

Two peaks of ^3H -labelled material with the chromatographic properties of an InsP_4 were detected on Partisphere SAX HPLC. These were desalted. The one which eluted first was mixed with authentic standards of D - and/or L - $\text{Ins}(1,2,3,4)\text{P}_4$ and $\text{Ins}(1,2,5,6)\text{P}_4$, obtained from ^{32}P -labelled germinating mung

Figure 7 Polypore Pb^{2+} HPLC separation of the polyol products of periodate oxidation, reduction and dephosphorylation of an aleurone InsP_4

A ^3H InsP_4 fraction, one of two detected in ^3H Ins -labelled aleurone layers, and which eluted after both $\text{Ins}(3,4,5,6)\text{P}_4$ and $\text{Ins}(1,2,3,4)\text{P}_4$ on Partisphere SAX HPLC (results not shown), was desalted and subjected to periodate oxidation, reduction and dephosphorylation to yield ^3H polyols. The products were mixed with authentic standards of ^{14}C inositol, ^{14}C xylitol and D - ^{14}C glucitol and resolved on a Brownlee Polypore Pb^{2+} column eluted with water at a flow rate of 0.2 ml/min. The injection volume was 50 μl . Fractions (0.5 min) were collected and the ^3H and ^{14}C contents determined by dual-label scintillation counting. The positions of elution of unlabelled polyol standards were determined in a parallel HPLC run by measurement of UV absorbance at 190 nm (all steps as described in the Materials and methods section). $\circ$, ^3H ; $\bullet$, ^{14}C ; solid line, UV absorbance in arbitrary units.

bean, and resolved on a Partisphere SAX column eluted with a gradient optimized for separation of InsP_4 s [12]. Under such conditions the ^3H -labelled peak co-eluted precisely with D - and/or L - ^{32}P $\text{Ins}(1,2,3,4)\text{P}_4$ and before ^{32}P $\text{Ins}(1,2,5,6)\text{P}_4$ (Figure 6), and in a separate run (results not shown) after $\text{Ins}(3,4,5,6)\text{P}_4$ obtained from ^{14}C Ins -labelled *Spirodela polyrhiza* [8]. This peak also appeared to be resistant to mild acid treatment, in as much that such treatment did not generate any new peaks when the products were applied to Partisphere SAX (results not shown). Such resistance to mild acid treatment is suggestive of complete substitution of positions 1, 2 and 3. The identity of the major InsP_4 was pursued by vigorous periodate oxidation, reduction and dephosphorylation. Although the polyol products resolved on Polypore Pb^{2+} HPLC contained several peaks, only one co-eluted with an authentic standard of those polyols which are the expected products of periodate oxidation, reduction and dephosphorylation of InsP_4 s (results not shown). Thus the presence of some ^3H -labelled material which co-eluted precisely with altritol, and not with *myo*-inositol, arabitol, xylitol, glucitol or iditol, unambiguously identifies the parent InsP_4 as D - and/or L - $\text{Ins}(1,2,3,4)\text{P}_4$ without any reference to chromatography of the parent compound on Partisphere SAX HPLC. Nevertheless, the precise co-elution of the ^3H -labelled peak with D - and/or L - ^{32}P $\text{Ins}(1,2,3,4)\text{P}_4$ on Partisphere SAX HPLC is entirely consistent with the identification of altritol in the products of periodate oxidation, reduction and dephosphorylation.

The identification of the second peak of ^3H Ins -labelled material was pursued by periodate oxidation, reduction and dephosphorylation. Apart from a trace of material which eluted on Polypore Pb^{2+} HPLC shortly after an internal standard of ^{14}C inositol, the single major product co-eluted precisely with a standard of D - ^{14}C glucitol, after D - ^{14}C xylitol and before iditol (Figure 7). The enantiomerism of the glucitol obtained has not yet been determined; nevertheless, the generation of glucitol on oxidation, reduction and dephosphorylation of the parent compound identifies the second and minor InsP_4 eluted on Partisphere

Figure 8 Adsorbosphere SAX HPLC of aleurone InsP_5 s

A perchloric acid extract of [^3H]inositol-labelled aleurone tissue was neutralized, and an aliquot was mixed with authentic standards of [^{32}P]Ins(1,3,4,5,6) P_5 , D/L-[^{32}P]Ins(1,2,4,5,6) P_5 and a trace of D/L-[^{32}P]Ins(1,2,3,4,5) P_5 and applied to an Adsorbosphere SAX HPLC column. The column was eluted according to [9,22], and radioactivity was determined by on-line 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 at a ratio of 1:4 (v/v). Scintillation events were counted with an integration interval of 15 s. ○, ^3H ; ●, ^{32}P .

SAX as D- and/or L-Ins(1,2,5,6) P_4 , the D-enantiomer of which has previously been identified in germinating mung bean [12].

InsP_5 s

Partisphere SAX HPLC [8] of extracts obtained from [^3H]Ins-labelled tissue revealed three peaks of InsP_5 (Figure 1). In some HPLC runs a slight shoulder was more clearly defined on the tail of the final peak. A tissue extract was mixed with authentic standards of [^{32}P]Ins(1,3,4,5,6) P_5 , D/L-[^{32}P]Ins(1,2,4,5,6) P_5 and a trace of D/L-[^{32}P]Ins(1,2,3,4,5) P_5 , obtained either directly from ^{32}P -labelled turkey erythrocytes or by acid-catalysed phosphate migration of the [^{32}P]Ins(1,3,4,5,6) P_5 obtained therefrom, and resolved on Partisphere SAX HPLC. We observed (results not shown) a minor peak which co-eluted with [^{32}P]Ins(1,3,4,5,6) P_5 and two larger peaks which eluted respectively with D/L-[^{32}P]Ins(1,2,3,4,5) P_5 and between D/L-[^{32}P]Ins(1,2,3,4,5) P_5 and D/L-Ins(1,2,4,5,6) P_5 . Given that all of the InsP_5 s which can theoretically be resolved on non-chiral HPLC systems were resolved, we identified the two major InsP_5 s as D- and/or L-Ins(1,2,3,4,5) P_5 (mixture of enantiomers undefined) and Ins(1,2,3,4,6) P_5 , and the minor peak as Ins(1,3,4,5,6) P_5 . There was also a small amount of (most likely) D- and/or L-Ins(1,2,4,5,6) P_5 in the tail of the final peak. This conclusion was confirmed by Adsorbosphere SAX HPLC according to [8,22]. The order of elution of InsP_5 s on such columns is Ins(1,2,3,4,6) P_5 , D/L-Ins(1,2,3,4,5) P_5 , D/L-Ins(1,2,4,5,6) P_5 /Ins(1,3,4,5,6) P_5 [22]. Consequently, the elution of two major peaks of [^3H]Ins P_5 before, and a third minor peak with, D/L-[^{32}P]Ins(1,2,4,5,6) P_5 /[^{32}P]Ins(1,3,4,5,6) P_5 (Figure 8) confirms the identification of the two principal ^3H -labelled peaks as Ins(1,2,3,4,6) P_5 and D- and/or L-Ins(1,2,3,4,5) P_5 . The small

amount of D- and/or L-[^{32}P]Ins(1,2,3,4,5) P_5 evident in the Figure is a consequence of limited *trans*-migration of phosphate on acid treatment [8].

Interestingly, Ins(1,3,4,5,6) P_5 , which is the precursor of InsP_6 in *Spirodela polyrhiza* [8,9] and *Dictyostelium discoideum* [22], was only a minor component of aleurone InsP_5 s. Of the others, Ins(1,2,3,4,6) P_5 has been reported as a minor component in soybean [14], whereas D- and/or L-Ins(1,2,3,4,5) P_5 (enantiomers undefined) and D- and/or L-Ins(1,2,4,5,6) P_5 (predominantly the L-enantiomer) were detected in germinating mung beans [13]. D- and/or L-Ins(1,2,4,5,6) P_5 was the major InsP_5 in soybean. We have also detected what appears to be Ins(1,3,4,5,6) P_5 in germinating mung beans [8].

InsP_6

The most strongly labelled product of [^3H]inositol metabolism was tentatively identified as InsP_6 by nature of the late elution of this peak on Partisphere SAX columns (Figure 1). The precise co-elution of this peak with a standard of [^{14}C]Ins P_6 , obtained from *Spirodela polyrhiza* [8], on both Partisphere SAX and Adsorbosphere SAX columns (results not shown) is entirely consistent with this identification. We also observed a peak, minor in size relative to InsP_6 , which eluted on both Partisphere SAX and Adsorbosphere SAX columns substantially later than InsP_6 . The most likely identity of this peak is that of an inositol pyrophosphate. Such compounds have been characterized in animal cells [22].

DISCUSSION

In contrast to animal systems, in which many inositol phosphates of all levels of phosphorylation have been characterized in a rigorous stereochemical and enantiomeric manner, comprehensive analyses of the inositol phosphate content of plants are lacking. This represents a serious impediment to progress in plant inositol phosphate research. Many studies report the presence of inositol phosphates of all degrees of phosphorylation, and these are likely to include intermediates of InsP_6 synthesis and breakdown. Inositol phosphates of several classes have been rigorously identified in only a few plant tissues. D- and L-Ins(1,2,3,4) P_4 , Ins(1,2,5,6) P_4 , D- and L-Ins(1,2,4,5,6) P_5 and D- and/or L-Ins(1,2,3,4,5) P_5 have all been identified in germinating mung bean seedlings [12,13]. Ins(1,3,4,5,6) P_5 and Ins(1,2,3,4,6) P_5 represented less than 4% of total InsP_5 in [^3H]Ins- or [^{32}P]Ins-labelled preparations. Ins(1,3,4,5,6) P_5 is present in soybean but, along with D- and/or L-Ins(1,2,4,5,6) P_5 and Ins(1,2,3,4,6) P_5 , is only a minor component compared with D- and/or L-Ins(1,2,3,4,5) P_5 [14].

In this context, the work of Loewus and co-workers [10,11] on the stereochemistry of the cyclization of glucose 6-phosphate deserves attention, as the rigorous characterization of the enantiomerism of the products by gas chromatography and the subsequent discovery of *myo*-inositol kinase, which yields the same product, predates much of the current literature. Similarly, the characterization by Tomlinson and co-workers, Lim and Tate, and Irving and co-workers of the routes and products of InsP_6 breakdown *in vitro* by plant and fungal phytases, reviewed in [15], represents most of what is known about the stereo-specificity of inositol phosphate metabolism in plants. It is these latter studies which are perhaps of most relevance to the work presented here.

A crude preparation of phytase from wheat bran, the substrate specificity of which was characterized in detail by Tomlinson and Ballou [24,25], was further characterized by Johnson and Tate [26] and subsequently resolved into two fractions by Lim and

Tate [27,28]. The crude enzyme characterized by Tomlinson and Ballou [24,25] and the F1 fraction of the two activities resolved on DEAE-cellulose chromatography by Lim and Tate [27,28] yielded the same products from InsP_6 [15], perhaps indicating that the F2 fraction was a relatively minor component in the latter authors' preparation. Thus the principal site of F1 attack is the D-4 phosphate, yielding $\text{Ins}(1,2,3,5,6)\text{P}_5$, since further dephosphorylation yielded $\text{Ins}(1,2,3)\text{P}_3$ and $\text{Ins}(1,2,6)\text{P}_3$ [25].

Only two InsP_4 products were characterized with any certainty [25,26,29]: a minor dextrorotatory component, $\text{Ins}(1,2,3,6)\text{P}_4$, which is identical to the product of F2 phytase action on $\text{Ins}(1,2,3,5,6)\text{P}_5$ and $\text{Ins}(1,2,3,4,6)\text{P}_5$; and a major laevorotatory product, $\text{Ins}(1,2,5,6)\text{P}_4$. $\text{Ins}(1,2,6)\text{P}_3$ and $\text{Ins}(1,2,3)\text{P}_3$ were identified in the products of crude phytase action on InsP_6 [25]. Similarly, Davis (cited in [15]), who studied the pathway of InsP_6 breakdown by peanut phytase, showed that the pathway of breakdown was the same as that for the crude wheat phytase. $\text{Ins}(1,2,3)\text{P}_3$ alone was obtained in the products of F2 phytase attack on InsP_6 [28]. $\text{Ins}(1,2)\text{P}_2$ and/or $\text{Ins}(2,3)\text{P}_3$ were identified in the products of crude phytase action on InsP_6 [25], and it has been shown that wheat-bran phytase yields only a single InsP , $\text{Ins}2\text{P}$. [15].

Some of the above work was repeated [30] using a commercially available preparation of wheat phytase. The author was able to confirm much of these conclusions by NMR, but in a departure from the above also reported the formation of $\text{Ins}(1,5,6)\text{P}_3$ from $\text{Ins}(1,2,5,6)\text{P}_4$.

In contrast with the foregoing, the F2 fraction of wheat-bran phytase generated three chromatographically distinct peaks of InsP_5 [27,28]: $\text{Ins}(1,3,4,5,6)\text{P}_5$, $\text{Ins}(1,2,3,5,6)\text{P}_5$ (which is the major product of crude wheat-bran phytase action) and $\text{Ins}(1,2,3,4,6)\text{P}_5$. Further attack on the latter two isomers yielded the same product, $\text{Ins}(1,2,3,6)\text{P}_4$, which is the same isomer as the minor InsP_4 component produced by crude wheat-bran phytase. A single InsP_3 product was identified as $\text{Ins}(1,2,3)\text{P}_3$, again in common with one of the products of crude phytase. Further breakdown yielded a mixture of enantiomers, $\text{Ins}(2,3)\text{P}_2$ and $\text{Ins}(1,2)\text{P}_2$, with the latter predominating, and penultimately $\text{Ins}2\text{P}$ and then *myo*-inositol.

Returning to the present work, there is a striking similarity between the identities of the inositol phosphates, albeit in some cases stereoisomers and not enantiomers, obtained from [^3H]Ins-labelled barley aleurone layers and the products of wheat-bran phytase action *in vitro*. The most parsimonious explanation is that the majority of the inositol phosphates identified in the present study are indeed the products of phytase action *in vivo*. A significant point of departure is the identification of D- and/or L- $\text{Ins}(1,4)\text{P}_2$. Given the precedent of the identification of $\text{Ins}(1,4)\text{P}_2$ in *Spirodela polyrhiza* [8] and the description of a pathway of synthesis of InsP_6 in that plant which, in contradiction to InsP_6 synthesis in *Dictyostelium* [30a], does not use the enantiomer of $\text{Ins}(1,4)\text{P}_2$, [i.e. $\text{Ins}(3,6)\text{P}_2$], the identification of D- and/or L- $\text{Ins}(1,4)\text{P}_2$ in barley aleurone suggests that this compound is neither a product of phytase action *in vivo* nor an intermediate in the pathway of InsP_6 synthesis. A caveat must be introduced here in that, although in the earlier metabolic study of the order of addition of phosphates to the inositol moiety of InsP_6 in *Spirodela* [9] we were unable to determine whether the 4-phosphate was added before the 6-phosphate, or vice versa, the structural data in the previous paper [8] does not allow for synthesis of $\text{Ins}(3,4,6)\text{P}_3$ by 4-phosphorylation of $\text{Ins}(3,6)\text{P}_2$, since we did not find $\text{Ins}(3,6)\text{P}_2$ in *Spirodela*. Significantly, with the likely exception of $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$, few of the inositol polyphosphates identified in *Spirodela* match those identified in barley aleurone tissue. Moreover, the preponderance

of $\text{Ins}(1,2,3,4,6)\text{P}_5$ and D- and/or L- $\text{Ins}(1,2,3,4,5)\text{P}_5$ over $\text{Ins}(1,3,4,5,6)\text{P}_5$ in barley aleurone when compared with the great excess of $\text{Ins}(1,3,4,5,6)\text{P}_5$ over other isomers in *Spirodela polyrhiza* suggests that we are looking at tissues carrying out two fundamentally different processes.

It is not entirely clear from the data presented here whether the pattern of inositol phosphates identified can be attributed to one or more of the isoforms of phytase activity identified in wheat bran. Nevertheless, the large amounts of $\text{Ins}(1,2,3,4,6)\text{P}_5$ present in the InsP_5 fraction, and the predominance of D- and/or L- $\text{Ins}(1,2,3,4)\text{P}_4$ and $\text{Ins}(1,2,3)\text{P}_3$ in their respective classes, suggests very strongly that the contribution of the F2 isoform is significant. The physiological significance of this is not immediately evident. There is much confusion in the literature regarding the control of synthesis, activation and secretion of the numerous isoforms of acid phosphatase in the aleurone tissue of cereals [31–35]. Activities such as these have commonly been shown to attack sodium phytate. Activities and particular isoforms have been located in the cytosol, and in association with the cell wall, the endoplasmic reticulum and protein bodies. They have variously been shown to be gibberellin-sensitive or -insensitive. However, we should be careful to note that the description of phytate mobilization in much of the aleurone work assumes that enzyme activities with rather poorly defined substrate specificity are responsible for the mobilization of phytate reserves. There is no indication of how the different isoforms relate to the wheat-bran phytases studied by Lim and Tate and other workers [15,24–29]. A biochemical description of phytate reserve mobilization will ultimately require a stereochemical description of the products of phytate breakdown *in vivo*. It would, therefore, be particularly interesting to characterize the inositol phosphate products of 'phytase' action in the aleurone cell at a stage when InsP_6 breakdown can be shown to predominate over InsP_6 synthesis. More importantly, perhaps, we cannot be sure that the synthesis and metabolism of InsP_6 observed in the present study is anything other than a facet of cytosolic InsP_6 metabolism, possibly unrelated to the assumed storage function of phytate in plants. Many of the inositol phosphates identified *in vivo* in barley aleurone tissue have counterparts, the functions of which are equally undefined, in the animal kingdom.

The identification of D- and/or L- $\text{Ins}(1,2,3,4)\text{P}_4$ and D- and/or L- $\text{Ins}(1,2,5,6)\text{P}_4$ in barley aleurone, and of the isomers D- and L- $\text{Ins}(1,2,3,4)\text{P}_4$ and D- $\text{Ins}(1,2,5,6)\text{P}_4$ in germinating mung bean [12], may go some way to rationalizing the synthetic or degradative status of these isomers in plants. These isomers may be universal products of InsP_6 metabolism or phytate reserve breakdown in plant storage tissues. Thus Davis (cited in [15]) described a pathway of InsP_6 breakdown by peanut phytase identical to that described in the *in vitro* work discussed above and very similar to that inferred in the present study. An element of caution must be introduced, however, in that there is no consensus for the pattern of InsP_5 isomers present in the four plant systems, i.e. mung bean [12,13], soybean [14], *Spirodela polyrhiza* [8] and barley aleurone (present study), which have been characterized to date. This may be a consequence of differences in the balance between synthesis and breakdown and different physiological statuses in the four systems, only one of which is a single cell type. Interestingly, Stephens and co-workers [13], through an analysis of the rate constants of InsP_6 synthesis from different InsP_5 isomers and of the isomers in germinating mung beans, have suggested that the major isomer, L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ [79 % of the D- and/or L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ fraction], is probably an intermediate in a dephosphorylation pathway, since it is a member of a pair of enantiomers [D- and L- $\text{Ins}(1,2,4,5,6)\text{P}_5$] which in a racemic mixture were least readily

phosphorylated *in vitro* by the InsP_5 hydroxykinase activity detected in germinating mung bean. Should this proposition be upheld by investigation, then it will be necessary to accommodate a novel class of phytase, since the D-1 phosphate is released late in the sequence of intermediates by which InsP_6 is dephosphorylated by the plant phytases studied to date. The very low levels of D- and/or L- $\text{Ins}(1,2,4,5,6)\text{P}_5$ in aleurone tissue, and the apparent degradative status of the other InsP_5 s detected in this tissue *in vivo*, argue against the predominance of a D-1 phytase in aleurone tissue.

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