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myo-Inositol pentakisphosphates

Structure, biological occurrence and phosphorylation to *myo*-inositol hexakisphosphate

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1. Standard and high-performance anion-exchange-chromatographic techniques have been used to purify *myo*-[^{3}H]inositol pentakisphosphates from various *myo*-[^{3}H]inositol-prelabelled cells. Slime mould (*Dictyostelium discoideum*) contained 8 μM-*myo*-[^{3}H]inositol 1,3,4,5,6-pentakisphosphate, 16 μM-*myo*-[^{3}H]inositol 1,2,3,4,6-pentakisphosphate and 36 μM-D-*myo*-[^{3}H]inositol 1,2,4,5,6-pentakisphosphate [calculated intracellular concentrations; Stephens & Irvine (1990) Nature (London) **346**, 580–583]; germinating mung-bean (*Phaseolus aureus*) seedlings contained both D- and L-*myo*-[^{3}H]inositol 1,2,4,5,6-pentakisphosphate (which was characterized by ^{31}P and two-dimensional proton n.m.r.) and D- and/or L-*myo*-[^{3}H]inositol 1,2,3,4,5-pentakisphosphate; HL60 cells contained *myo*-[^{3}H]inositol 1,3,4,5,6-pentakisphosphate (in a 500-fold excess over the other species), *myo*-[^{3}H]inositol 1,2,3,4,6-pentakisphosphate and D- and/or L-*myo*-[^{3}H]inositol 1,2,4,5,6-pentakisphosphate; and NG-115-401L-C3 cells contained *myo*-[^{3}H]inositol 1,3,4,5,6-pentakisphosphate (in a 100-fold excess over the other species), D- and/or L-*myo*-[^{3}H]inositol 1,2,4,5,6-pentakisphosphate, *myo*-[^{3}H]inositol 1,2,3,4,6-pentakisphosphate and D- and/or L-*myo*-[^{3}H]inositol 1,2,3,4,5-pentakisphosphate. 2. Multiple soluble ATP-dependent *myo*-inositol pentakisphosphate kinase activities have been detected in slime mould, rat brain and germinating mung-bean seedling homogenates. In slime-mould cytosolic fractions, the three *myo*-inositol pentakisphosphates that were present in intact slime moulds could be phosphorylated to *myo*-[^{3}H]inositol hexakisphosphate: the relative first-order rate constants for these reactions were, in the order listed above, 1:8:31 respectively (with first-order rate constants in the intact cell of 0.1, 0.8 and 3.1 s^{-1}, assuming a cytosolic protein concentration of 50 mg/ml), and the K_m values of the activities for their respective inositol phosphate substrates (in the presence of 5 mM-ATP) were 1.6 μM, 3.8 μM and 1.4 μM. At least two forms of *myo*-inositol pentakisphosphate kinase activity could be resolved from a slime-mould cytosolic fraction by both pharmacological and chromatographic criteria. Rat brain cytosol and a soluble fraction derived from germinating mung-bean seedlings could phosphorylate *myo*-inositol D/L-1,2,4,5,6-, D/L-1,2,3,4,5-, 1,2,3,4,6- and 1,3,4,5,6-pentakisphosphates to *myo*-inositol hexakisphosphate: the relative first-order rate constants were 57:27:77:1 respectively for brain cytosol (with first-order rate constants in the intact cell of 0.0041, 0.0019, 0.0056 and 0.000073 s^{-1} respectively, assuming a cytosolic protein concentration of 50 mg/ml) and 1:11:12:33 respectively for mung-bean cytosol (with first-order rate constants in a supernatant fraction with a protein concentration of 10 mg/ml of 0.0002, 0.0022, 0.0024 and 0.0066 s^{-1} respectively).

INTRODUCTION

Of the six isomers of InsP_5, Ins(1,3,4,5,6)P_5 was the first to be characterized in biological extracts (Johnson & Tate, 1969). This InsP_5 isomer has since been identified as the major InsP_5 in bovine brain and platelets (Phillippy & Bland, 1988; Mayr, 1988), but it was the minor InsP_5 in extracts of soya bean (Phillippy & Bland, 1988). Proven biological functions for InsP_5 are few. B. B. Biswas *et al.* (1978) have proposed that Ins(1,3,4,5,6)P_5 is the intermediate in both the synthesis and degradation of InsP_6 in mung beans, by serving either as a substrate for, or the product of (in the 'forward direction' by their definition) an InsP_6/ADP phosphotransferase. In erythrocytes of a number of amphibians and the majority of birds Ins(1,3,4,5,6)P_5 serves as a modulator of the oxygen affinity of haemoglobin (much as 2,3-diphosphoglycerate does in mammals; Bartlett, 1982). The ease with which Ins(1,3,4,5,6)P_5 can be isolated from avian erythrocytes has resulted in it becoming commercially available; hence it has been tested in more biological assays than any of the other isomers of InsP_5. Reports of the ability of Ins(1,3,4,5,6)P_5 to excite neurons in the brain stems of rats (Vallejo *et al.*, 1987) and potently to inhibit mammalian aldolase A (Koppitz *et al.*, 1986) and Ins(1,3,4,5)P_4 3-phosphatase (Hughes & Shears, 1990) have been published. In contrast with Ins(1,3,4,5,6)P_5, the lack of availability of the other InsP_5 isomers has meant that they have received less attention; as a result, little is known of the specificity of the effects of Ins(1,3,4,5,6)P_5 on the various processes described above, and nothing is known of the biological properties of any of the other five InsP_5 isomers.

Although InsP_6 is the most naturally abundant (in terms of mass in the biosphere) inositol phosphate, and many functions have been ascribed to it (mainly in its capacity as a storage molecule; Williams, 1970), its synthesis is ill-understood. An activity has been purified from mung beans that can transfer a phosphate from ATP to Ins(1,3,4,5,6)P_5 (see above; S. Biswas *et al.*, 1978), yielding InsP_6; however, the ability of a crude homogenate to phosphorylate any of the other InsP_5 isomers was not investigated. More recently it has been reported that a ^{3}H-labelled molecule, with the chromatographic properties of an InsP_5 (extracted from [^{3}H]Ins-prelabelled adrenal glomerulosa cells) could be converted into a [^{3}H]InsP_6-like compound when injected into *Xenopus* oocytes (Ji *et al.*, 1990). It was suggested that an InsP_5 kinase activity was responsible for catalysing the reaction, although neither its substrate specificity nor product

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were defined. Some of the metabolic pathways in the slime mould *Dictyostelium discoideum* that make and break down InsP_6 have recently been characterized (Stephens & Irvine, 1990). Evidence was presented that three InsP_5 isomers could be detected in intact amoebae, all of which were substrates for putative InsP_5 hydroxy kinase(s), but only one of which was the precursor *de novo* of InsP_6. The experiments described in the present paper were aimed at establishing in *Dictyostelium* the specificity and properties of the InsP_5 hydroxy kinase (or if indeed there are multiple forms of the enzyme), and whether similar activities can be identified in other plant and animal cells. We also present for the first time some of the detailed strategies and supporting data which enabled the InsP_5 isomers in the amoebae to be identified and which were used to characterize the InsP_5 isomers found in a number of other cellular systems.

We should note the nomenclature procedure that we have followed here. Under a recent IUPAC suggestion, the abbreviation Ins may be used for D-*myo*-inositol. This abbreviation is not followed in this paper; here, Ins stands for *myo*-inositol with no enantiomeric determination. As *myo*-inositol has a plane of symmetry down the 2/5-OH axis, there are two InsP_5 isomers which are *meso* compounds: Ins(1,3,4,5,6)P_5 and Ins(1,2,3,4,6)P_5. However, the other four InsP_5 isomers form two enantiomeric pairs. Thus D-Ins(1,2,4,5,6)P_5 and D-Ins(2,3,4,5,6)P_5 may be called instead D- and L-Ins(1,2,4,5,6)P_5 respectively. Similarly, D-Ins(1,2,3,4,5)P_5 and D-Ins(1,2,3,5,6)P_5 are an enantiomeric pair which may alternatively be called D- and L-Ins(1,2,3,4,5)P_5 respectively. Because of the emphasis on enantiomeric determinations in parts of this paper, we have used the latter system throughout. It should therefore be noted that D-Ins(2,3,4,5,6)P_5 and D-Ins(1,2,3,5,6)P_5 do not appear so named in this paper, but are instead referred to solely by their alternative names, L-Ins(1,2,4,5,6)P_5 and L-Ins(1,2,3,4,5)P_5 respectively. If the terms D- or L- are used, it is only for compounds whose enantiomeric configuration is known. For uncharacterized samples extracted from biological sources we use the designation D- and/or L-, which emphasizes that the InsP_5 in question may be either D- or L-, or a mixture of both in unknown proportions. However, samples generated by symmetrical chemical reactions (for example, non-enzymic phosphorylation or alkaline dephosphorylation) will inevitably be exactly 50:50 mixtures of enantiomers, and the designation D/L is used to describe these.

MATERIALS AND METHODS

Incubation of *Dictyostelium* with [^{3}H]Ins and [^{32}P]P_i

Dictyostelium discoideum (strain NC4) was co-cultured with *Klebsiella aerogenes* as described previously (Kay & Trevan, 1981). Cells were harvested into 20 mM-NaCl/20 mM-KCl/1 mM-$CaCl_2$/10 mM-Mes (pH 6.2, 22 °C) and freed from bacteria by differential centrifugation (Kay & Trevan, 1981). Amoebae harvested from half-cleared plates were incubated with [^{32}P]P_i or [^{3}H]Ins in the salt solution in which they were harvested at 2×10^7 cells/ml and shaken at 180 rev/min on an orbital mixer. Labelling reactions were quenched by pelleting the amoebae by centrifugation (5 min at 300 *g*), aspirating the medium and adding ice-cold 5% (v/v) $HClO_4$ (2 ml). After 5 min on ice, the acid-precipitated cellular debris was pelleted by centrifugation (bench-top, maximum setting) and the supernatants were neutralized with 2 M-KOH/0.1 M-Mes/25 mM-EDTA (approx. 0.5 ml) and stored at −80 °C. The acid extracts were separated on anion-exchange h.p.l.c. columns (see below).

Preparation of subcellular fractions from *Dictyostelium*

Amoebae harvested from half-cleared plates (see above) were resuspended at 3×10^8 cells/ml in 10% (v/v) glycerol/10 mM-Hepes/1 mM-EGTA/0.1 mM-phenylmethanesulphonyl fluoride (pH 7.5, 4 °C), then lysed by forcing them through a filter (Das & Henderson, 1983). The homogenates produced by filtration were mixed with 0.009 vol. of 1 M-Hepes (bringing the final overall pH to 7.0) and dithiothreitol (final concn. 1 mM). Supernatant fractions were prepared from these homogenates by centrifugation (1 h, 100000 *g*, 4 °C), and particulate fractions were prepared by resuspending the membrane pellet to the volume of the original lysate with lysis buffer (pH adjusted and dithiothreitol added). Cytosolic and particulate fractions prepared in this way were usually used immediately, although supernatants were successfully stored at −80 °C for up to 2 months. To enable assays to be run under first-order conditions with only 5–25% substrate utilization, cytosolic fractions were diluted into 25 mM-Hepes/1 mM-EGTA/1 mM-dithiothreitol/1 mg of BSA/ml (pH 7.0, 4 °C).

Development of *Dictyostelium*

AX2, an axenic laboratory strain of the cellular slime mould *Dictyostelium*, was cultured, harvested and developed as described previously (Watts & Ashworth, 1970; Stephens *et al.*, 1990). Cell lysates for enzyme assays were prepared in two ways: (*a*) as described by Kay (1979) for the cyclic AMP phosphodiesterase and glycogen phosphorylase assays (essentially 2×10^7 cells were scraped from the solid support, pelleted in a Microfuge, and the supernatant was removed and the pellet was solubilized with 1 ml of lysis buffer); or (*b*) as described by Stephens & Irvine (1990) for the [^{3}H]InsP_5 hydroxy kinase assays. Essentially, $(3–4) \times 10^8$ cells were scraped from the solid support, and samples containing 0.85×10^8 cells were pelleted in a Microfuge, the supernatant was removed and the cells were lysed into 1 ml of lysis buffer. Samples of these lysates were assayed for protein and then diluted to 1 mg of protein/ml with additional lysis buffer, and then diluted again into 25 mM-Hepes/1 mM-EGTA/1 mM-dithiothreitol/1 mg of BSA/ml before finally assaying the [^{3}H]InsP_5 hydroxy kinase activities as described above.

Acid extracts were prepared from 4×10^8 cells (one 14 cm-diameter culture plate, three plates for each time point) by scraping the cells into 10 ml of harvesting buffer and then pelleting them by centrifugation, removing the supernatant, adding 1.7 ml of 0.6 M-$HClO_4$, vortex-mixing and standing them on ice. After 5 min on ice, the acid extracts were mixed with Ins[^{32}P]P_6 (900 c.p.m., prepared from [^{32}P]P_i-labelled mung beans and containing less than 1 nmol of total phosphorus), then centrifuged for 5 min (at the maximum setting of a bench-top centrifuge), and the supernatant was neutralized with 2 M-KOH/0.1 M-Mes/25 mM-EDTA. The $KClO_4$ precipitate was compacted by centrifugation, and the supernatant was filtered and applied to a weak anion-exchange h.p.l.c. column (Partisphere, 25 cm). The column was eluted with a gradient (A = water, B = 2.5 M-NaH_2PO_4, pH 3.8) following the pattern: 0 min, 0% B; 15 min, 25% B; 50 min, 67% B; 51 min, 100% B; 56 min, 100% B (1.0 ml/min). The quality of the chromatography obtained was routinely checked by monitoring the absorbance (at 254 nm) of the eluate; fractions were collected every 1 min and counted for ^{32}P by Čerenkov counting techniques. Those fractions containing ^{32}P were pooled, desalted (Stephens & Downes, 1990) and assayed for both free inorganic phosphorus and total phosphorus (Baginski *et al.*, 1967; Bartlett, 1959, as modified by Galliard *et al.*, 1965). The InsP_6 concentration in the original extracts was calculated by assuming an inositol/phosphorus ratio of 1:6 and using the amount of ^{32}P finally recovered in the fractions assayed for phosphorus as an estimate of the recovery of InsP_6 through the extraction, purification and desalting steps (recoveries ranged from 55 to 80%). The data were finally expressed in terms of the amount of InsP_6

per mg of cellular protein by solubilizing the original $HClO_4$-precipitated cell debris in warm 1 M-NaOH and assaying for protein with BSA as a standard (Bradford, 1976).

Preparation of a soluble fraction from mung-bean seedlings

Mung beans were cleaned with 70% ethanol and then with distilled water and allowed to germinate in distilled water (approx. 1 g of mung beans to 10 ml of water) in the dark at room temperature. Mung-bean homogenates were prepared by germinating mung beans in water, then decoating and disrupting them (10 g wet wt.), first in a large rotating-blade homogenizer (30 s) and then with a Polytron (15 s) into 10 ml of 25 mM-Hepes/1 mM-EGTA/1 mM-dithiothreitol (pH 7.0, 4 °C). The homogenate was centrifuged (100000 *g*, 1 h, 4 °C) and the supernatant was used immediately.

Mammalian cell culture

NG-115-401L-C3 cells were cultured and labelled with [^{3}H]Ins for 3 days in 35 mm-diam. Petri dishes (1 mCi of [^{3}H]Ins/ml) in a medium of inositol-free Dulbecco's modified Eagle medium with 10% (v/v) dialysed foetal-calf serum, as described previously (Jackson *et al.*, 1987). Acid extracts were prepared from [^{3}H]Ins-prelabelled cells by sequentially aspirating the medium, adding ice-cold 5% (v/v) $HClO_4$ (2 ml), scraping the cells from the dish, pelleting the precipitated cellular debris by centrifugation and neutralizing the supernatant with 2 M-KOH/0.1 M-Mes/25 mM-EDTA.

HL60 cells were cultured in inositol-free RPMI medium (Gibco) with 10% foetal-calf serum either with or without [^{3}H]Ins (as described in French *et al.*, 1988). [^{3}H]Ins-prelabelled cells (after 4 days in the presence of [^{3}H]Ins) were pelleted by centrifugation, their medium was aspirated and ice-cold 5% $HClO_4$ was added (2 ml). After mixing, the cellular debris was pelleted by centrifugation and the supernatant was neutralized with 2 M-KOH/0.1 M-Mes/25 mM-EDTA.

Preparation of subcellular fractions from rat brain

Rat brain homogenates, particulate and cytosol fractions were prepared as described in Stephens *et al.* (1988*b*). One rat brain was routinely homogenized in 5 ml of buffer and the homogenate centrifuged at 100000 *g* for 45 min to produce a crude cytosol fraction and a membrane pellet. The membrane pellet was resuspended to the original volume of the homogenate with fresh buffer to yield a particulate fraction.

Resolution of InsP_5 isomers by anion-exchange h.p.l.c.

Mixtures of, or individual, radiolabelled InsP_5 isomers (prepared as described below) were separated on either weak or strong anion-exchange h.p.l.c. columns (Whatman). Partisphere WAX columns [which had been pre-equilibrated with buffer B: 0.5 M-$(NH_4)_2HPO_4$, adjusted to pH 3.2 with H_3PO_4, 22 °C] were eluted with a gradient based on water and buffer B (see above) to the pattern 0 min 0% B, 1 min 40% B, 60 min 70% B, 61 min 100% B, 70 min 100% B, at a flow rate of 1 ml/min. Fractions were collected and counted for radioactivity by standard liquid-scintillation counting techniques. Partisil 10SAX or Partisphere 5SAX columns were pre-equilibrated and eluted with 2.5 M-NaH_2PO_4 (pH 3.75 with NaOH, 22 °C) or 1.25 M-$(NH_4)_2HPO_4$ (pH 3.8 with H_3PO_4, 22 °C) respectively. The pattern of phosphate gradient employed with these columns was identical with that used with the WAX column described above.

Resolution of InsP_5 and InsP_6 by batch elution of anion-exchange resin

For this, 12 cm × 0.6 cm columns of Bio-Rad AG 1 × 8 (200–400 mesh; chloride form) were used (some columns were capped with a 5 mm-deep layer of HCl-washed sand; this resulted in a marginal decrease in the amount of the InsP_5 isomers eluted in the InsP_6 fractions and significantly prolonged the life of the columns by protecting against dehydration). Samples were loaded in 5 ml of water and washed on with a further 5 ml of water. InsP_5 isomers were eluted as follows: [^{3}H]Ins(1,2,3,4,6)P_5 and D/L-[^{3}H]Ins(1,2,3,4,5)P_5 with 5 × 10 ml of 0.65 M-HCl; D/L-[^{3}H]Ins(1,2,4,5,6)P_5 with 6 × 10 ml of 0.65 M-HCl; and [^{3}H]Ins(1,3,4,5,6)P_5 with 8 × 10 ml of 0.63 M-HCl. InsP_6 was eluted with 2 × 10 ml of 1.5 M-HCl. Representative profiles are shown in Fig. 1. The spill-over of InsP_5 into InsP_6 fractions was always less than 0.5%, and the loss of InsP_6 into InsP_5 fractions (which was assessed in most experiments by the addition of a portion of Ins[^{32}P]P_6 to each sample before application to the resin columns) was at worst 1.5%. The 10 ml fractions were all mixed with 10 ml of either Packard 299 or Zinsser Hi-salt scintillation fluid and counted for ^{3}H and/or ^{32}P radioactivity by standard dual-label liquid-scintillation counting techniques.

Preparation of standard InsP_5 isomers and InsP_6

Preparation of ^{3}H- or ^{32}P-labelled InsP_5 isomers and InsP_6 from mung beans. To prepare radiolabelled D- and/or L-Ins(1,2,3,4,5)P_5, D- and/or L-Ins(1,2,4,5,6)P_5 and InsP_8, [^{3}H]Ins or [^{32}P]P_i was added to the distilled water in which mung beans were germinated (see above and the Results section; typically, 0.5 mCi of [^{3}H]Ins or [^{32}P]P_i and a single mung bean were used). After 55–60 h the mung beans were removed from the residual water and homogenized with a glass-on-glass (studded pestle) hand homogenizer in 2 ml of ice-cold 5% $HClO_4$. The homogenized tissue was pelleted by centrifugation and the supernatant neutralized with octylamine/Freon (1:1, v/v; Sharpes & McCarl, 1982) before the addition of 50 μl of 0.1 M-EDTA. Radiolabelled compounds were purified from these extracts by h.p.l.c. (see above).

Preparation of Ins[^{32}P](1,3,4,5,6)P_5. Ins[^{32}P](1,3,4,5,6)P_5 was prepared from [^{32}P]P_i-labelled avian erythrocytes precisely as described previously (Stephens *et al.*, 1988*a*,*b*).

Preparation of high-specific-radioactivity [^{3}H]InsP_6 and [^{3}H]InsP_5 isomers. This was done by phosphorylation of [^{3}H]Ins as described previously (Cosgrove, 1980), except that high-specific-radioactivity [^{3}H]Ins (80–120 Ci/mmol; Amersham) was included in the reaction mixture without additional carrier and the quantities of the reagents employed were substantially decreased (Hawkins *et al.*, 1990). After the reaction was complete, the sample was neutralized, diluted and the [^{3}H]inositol polyphosphates were isolated by ion-exchange chromatography on an AG-1 (formate form) resin column and desalted by freeze-drying as described previously (Hawkins *et al.*, 1990).

[^{3}H]InsP_5 isomers and [^{3}H]InsP_6 were purified from the mixture by anion-exchange h.p.l.c. The sample was initially applied to a Partisil 10SAX column (eluted as described above) and the [^{3}H]InsP_6 peak (identified by its time of elution compared with that of authentic InsP_6 under identical circumstances or by inclusion of internal [^{32}P]InsP_6) was collected, desalted, re-applied to a Partisil 10SAX column and then eluted, collected and desalted again. [^{3}H]InsP_5 isomers were usually eluted in three major peaks; only the fractions containing the highest concentrations of ^{3}H in each peak were saved and desalted (corresponding to roughly 80% of the total ^{3}H in the peaks). The [^{3}H]InsP_5 isomers were each re-applied to a Partisphere WAX h.p.l.c. column and eluted as described above. The two [^{3}H]InsP_5 peaks which were eluted first from the Partisil 10SAX column were each eluted as single major peaks (containing 85–90% of the ^{3}H recovered), and were collected and desalted; the third

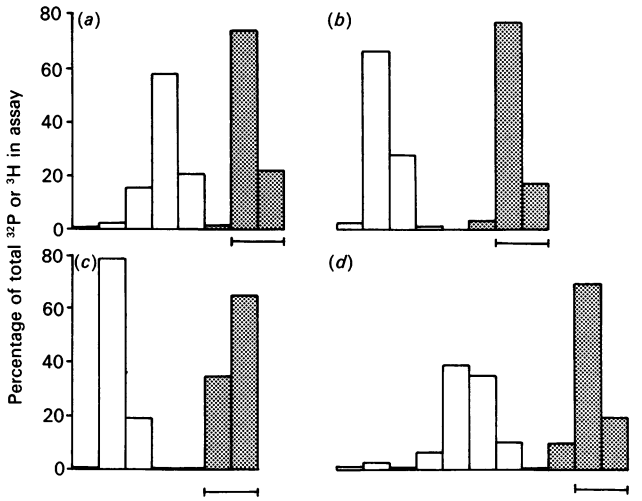

Fig. 1. Separation of [^{3}H]InsP_5s from [^{3}H]InsP_6 by batch elution from open-topped anion-exchange resin columns

Samples (20000 d.p.m.) of D/L-[^{3}H]Ins(1,2,4,5,6)P_5 (*a*), D/L-[^{3}H]Ins(1,2,3,4,5)P_5 (*b*), [^{3}H]Ins(1,2,3,4,6)P_5 (*c*) or [^{3}H]Ins-(1,3,4,5,6)P_5 (*d*) were mixed with 5000 d.p.m. of Ins[^{32}P]P_6 and applied to 12 cm × 0.6 cm columns of Bio-Rad AG 1 × 8 200–400 resin (chloride form) and then eluted with sequential 10 ml batches of 0.65 M-HCl (*a–c*) or 0.63 M-HCl (*d*) followed by two 10 ml batches of 1.5 M-HCl (marked with a horizontal line). The percentage of the total ^{3}H and ^{32}P (d.p.m.) eluted from the column in each 10 ml batch is shown in the diagram as a series of open bars and stippled bars respectively (the first batch at the extreme left). The results are typical of many hundreds of independently eluted columns. This quality of chromatography was maintained for at least 20–25 independent separations on a single column.

peak was eluted as two well-resolved peaks of ^{3}H, which were independently collected and desalted. The identities of the four species of [^{3}H]InsP_5 purified after phosphorylation of ^{3}H[Ins] were confirmed by co-injecting them with ^{32}P-labelled standard InsP_5 isomers (see below and above for preparation). Typically 15–20% of the starting ^{3}H would be recovered as pure [^{3}H]InsP_6 and 10–12% as pure [^{3}H]InsP_5 isomers. Prepared in this way, [^{3}H]Ins(1,3,4,5,6)P_5 and [^{3}H]Ins(1,2,3,4,6)P_5 are radiochemically pure, whereas D/L-[^{3}H]Ins(1,2,3,4,5)P_5 and D/L-[^{3}H]Ins-(1,2,4,5,6)P_5 are each racemic mixtures.

Preparation of [^{32}P]- or [^{3}H]-InsP_5 isomers by dephosphorylation of [^{32}P]- or [^{3}H]-InsP_6. [^{32}P]- or [^{3}H]-InsP_5 isomers were prepared by alkaline hydrolysis of [^{32}P]- or [^{3}H]-InsP_6 as described by Phillippy *et al.* (1987). Essentially, h.p.l.c.-pure InsP_6 (either ^{3}H- or ^{32}P-labelled) was dissolved in 0.5 ml of 50 mM-Na_2HPO_4 (pH 10.5 with NaOH) and then autoclaved (121 °C) for 3 h. After autoclaving, the sample was cooled, diluted to 2.5 ml and its pH adjusted to 7.0 with formic acid. InsP_5 isomers were resolved from this mixture by anion-exchange h.p.l.c. as described above (first on a Partisphere SAX column and then on a Partisphere WAX column). As with InsP_5 isomers obtained by phosphorylation of Ins (above), Ins(1,3,4,5,6)P_5 and Ins(1,2,3,4,6)P_5 can be obtained pure by this method, but D/L-Ins(1,2,4,5,6)P_5 and D/L-Ins(1,2,3,4,5)P_5 are both obtained as racemic mixtures.

D-[^{3}H]Ins(1,2,4,5,6)P_5 (as characterized by Irving & Cosgrove, 1972) was also prepared from [^{3}H]InsP_6 by dephosphorylation with a commercially available fungal phytase (*Aspergillus* phytase; Sigma). Optimal yields of InsP_5 were obtained when approx. 10–15% of InsP_6 phosphorus had been released as P_i. Under first-order conditions (with respect to InsP_6), incubation of InsP_6 with 0.0008 unit (units defined by Sigma) of *Aspergillus* phytase/ml in 20 mM-acetic acid (pH 5.0 with NaOH, 37 °C)/1 mg of BSA/ml resulted in 10% of the starting InsP_6 being dephosphorylated in 60 min at 37 °C. The assays were quenched with $HClO_4$ (final concn. 5%), precipitated protein was pelleted by centrifugation and the supernatant neutralized with 2 M-KOH/0.1 M-Mes/20 mM-EDTA. The reaction products were resolved by h.p.l.c. and the fractions containing the major InsP_5 were pooled and desalted.

Commercially available wheat-bran phytase was used to prepare ^{3}H- and ^{32}P-labelled D-Ins(1,2,3,5,6)P_5 from [^{3}H]- or [^{32}P]-InsP_6 (Tomlinson & Ballou, 1961; Lim & Tate, 1973). Assays contained [^{3}H]- or [^{32}P]-InsP_6 (final concentrations were typically in the 10 nM–10 μM range), 0.0006 unit of phytase/ml, 1 mg of BSA/ml and 20 mM-acetic acid (pH 5.0 with NaOH, 37 °C). After 60 min at 37 °C the assays were quenched and the reaction products resolved by h.p.l.c. as described above for the products of fungal phytase attack on InsP_6; 8–15% of the starting material (for [^{3}H]InsP_6) was typically recovered as InsP_5.

Preparation of large quantities of unlabelled InsP_5 isomers and InsP_6. InsP_6 was obtained as its barium salt from germinating mung beans as a by-product of the purification of InsP_5 isomers for n.m.r. analysis (see below). InsP_6 barium salt was converted into the sodium salt as described below [a 60-fold molar excess of Bio-Rad AG50 (H^+ form) was initially used to convert the InsP_6 into its acid form]. InsP_6 was also purified from commercially available InsP_6 preparations (Sigma) by chromatography on a column of Bio-Rad AG 1 × 8 200–400 anion-exchange resin (chloride form) as described previously (Cosgrove, 1969).

D/L-Ins(1,2,3,4,5)P_5, D/L-Ins(1,2,4,5,6)P_5, Ins(1,2,3,4,6)P_5 and Ins(1,3,4,5,6)P_5 were purified from an alkaline digest of InsP_6 essentially as described by Cosgrove (1969). Sodium phytate (5 g; Sigma) was dissolved in 100 ml of water and the pH was adjusted to 10.0 with HCl. Approx. 2×10^6 d.p.m. of [^{32}P]InsP_6 (prepared from ^{32}P-labelled mung beans; see above) was added to act as a marker for the presence of inositol phosphates in subsequent steps. The sample was autoclaved at 120 °C for approx. 60 min. The sample was then diluted to 150 ml with water and the pH adjusted to 7.0 with HCl. The sample was separated on a 60 cm × 2 cm column of Bio-Rad AG 1 × 8 (200–400 mesh; chloride form) by using an approximately linear gradient formed by the progressive mixing of 2.0 litres of 0.3 M-HCl with 2.0 litres of 1.5 M-HCl. The initial flow rate was approx. 110 ml/h and fractions were collected every 3 min. The fractions were analysed for the presence of inositol phosphates by measuring their ^{32}P content (Čerenkov radiation). The fractions corresponding to two broad InsP_5 peaks (Cosgrove, 1969) were combined and the pH was adjusted to 5.0 with $Ba(OH)_2$. The barium-InsP_5 precipitate was allowed to settle under gravity and washed with water. The first-eluted InsP_5 peak consists of a mixture of Ins(1,2,3,4,6)P_5 and D/L-Ins(1,2,3,4,5)P_5, and the second-eluted InsP_5 peak consists of a mixture of Ins(1,3,4,5,6)P_5 and D/L-Ins(1,2,4,5,6)P_5 (Cosgrove, 1969). These two samples were further resolved by chromatography on an identical AG1 (chloride form) column (see above) by isocratic elution with 0.48 M-HCl (Cosgrove, 1969). Each InsP_5 isomer was finally obtained in > 90% purity by three successive rounds of chromatography, with isocratic elution with 0.48 M-HCl in each case. The final washed barium salts of InsP_5 isomers were converted into the sodium salts by treatment with a 60-fold molar excess of Bio-Rad AG50 (H^+ form) resin and then neutralization with NaOH.

Ins(1,3,4,5,6)P_5 was also purified by h.p.l.c. with a preparative-scale Partisil 10-SAX h.p.l.c. column (the conditions used with analytical 10-SAX columns were scaled up) from commercially available preparations of Ins(1,3,4,5,6)P_5 (Calbiochem).

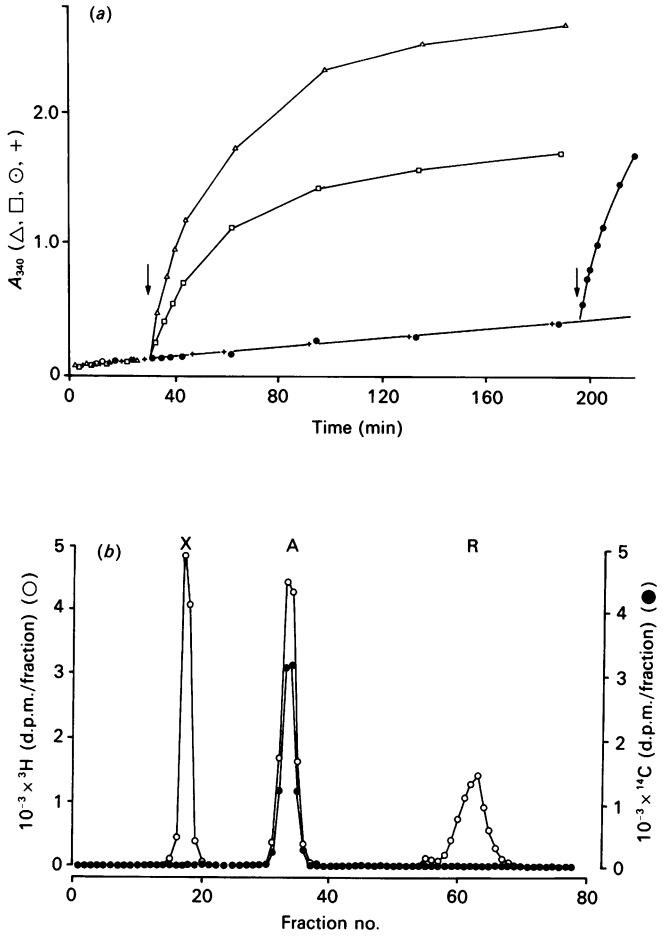

Fig. 2. Oxidation of L- but not D-arabitol by L-iditol dehydrogenase from yeast

(*a*) Four 1 ml quartz cuvettes, containing 950 μl of 100 mM-Tris.HCl, 20 mM-β-NAD^+ and 9.5 units of L-iditol dehydrogenase/ml (pH 8.3), were equilibrated at 25 °C. The A_{340} of each cuvette was monitored periodically. After 30 min, 50 μl of D-arabitol (+, final concn. 500 μM), L-arabitol (△, final concn. 1 mM; □, final concn. 500 μM), final concn.) or water (+) was added to the cuvettes and mixed. After 200 min, a further 5 μl of L-arabitol (final concn. 1 mM) was added to the cuvette to which water had initially been added (⊙). Only approx. 50% (range 30–65%) of the L-arabitol preparations appeared to be oxidized. This appeared to result from the oxidation process achieving equilibrium under the conditions of the assay, because addition of fresh substrate to a blank assay that had been incubated for 200 min (⊙) resulted in an initial rate of oxidation that was 93% of that obtained after 30 min of incubation. Furthermore, if the products that had accumulated after 2 h of incubation were purified and incubated with L-iditol dehydrogenase for a second time, then approx. 50% of the L-arabitol that had remained was again oxidized and the xylulose and ribulose that had remained (see below) generated some arabitol on incubation with L-iditol dehydrogenase. (*b*) An assay identical with that described in (*a*) above (□), except that it contained D-[^{14}C]arabitol and L-[^{3}H]arabitol, was quenched by boiling for 120 min after the addition of the substrate mixture. The sample was processed precisely as described previously (Stephens *et al.*, 1988*a*) and applied to a cation-exchange resin column in the Pb^{2+} form (see Stephens *et al.*, 1989). Fractions were collected every 1 min (0.2 ml) and counted for ^{3}H and ^{14}C radioactivity by standard dual-label liquid-scintillation-counting techniques; 85% of the ^{14}C and 60% of the ^{3}H was recovered. The elution positions of xylulose (X), arabitol (A) and ribulose (R) standards in a parallel run are marked (pure solutions of arabitol, ribulose and xylulose were independently injected and their progress through the system was monitored with an on-line differential refractomer, as described by Stephens *et al.*, 1988*a*). The elution times of the standards are corrected for the lag between the refractometer and fraction collector.

Inositol phosphates were stored either dry or in aqueous solution (pH 7.0) at −20 °C.

N.m.r. analysis

To prepare large quantities of InsP_5 for n.m.r. analysis, 25 g of mung beans were germinated for 60 h (as described above) and then homogenized in ice-cold 5% $HClO_4$ with two 10 s bursts in a rotating-blade homogenizer. The debris was pelleted by centrifugation (5000 *g*, 10 min, 4 °C) and the pH of the supernatant was adjusted to 6.0 with $Ba(OH)_2$. The precipitate was collected by centrifugation, and the supernatant was discarded, replaced with a similar volume of water, mixed and centrifuged again. The washed precipitate was mixed with 20 ml of Bio-Rad AG-50 resin (H^+ form). After standing at 4 °C for 60 min, the resin was mixed with 200 ml of water and separated from the liquid phase by filtration through a glass sinter. The pH of the filtrate was adjusted to 7.0 with NaOH. A total of 0.76 mmol of phosphorus was recovered at this point. The sample was mixed with a defined collection of [^{32}P]InsP_4s, [^{32}P]InsP_5s and [^{32}P]InsP_6 derived from a ^{32}P-labelled mung-bean preparation (see above and below) and applied to a 62 cm × 2 cm column of Bio-Rad AG 1 × 8 (200–400 mesh; chloride form) at 2.5 ml/min. The column was eluted with a gradient which started at 0 min at 0.12 M-HCl, and rose linearly over 4 litres to 1.2 M-HCl, at which point the eluent was switched to 2 M-HCl for a further 1.8 litres; 24 ml fractions were collected throughout. As fractions emerged from the column they were counted for ^{32}P radioactivity and stored at 4 °C in batches of 25. ^{32}P-containing fractions were pooled and precipitated with $Ba(OH)_2$ as described above. The more retained InsP_5 peak was converted into its acid form, neutralized with NaOH and quantified by phosphate assay (20.6 μmol of InsP_5 was recovered) before final submission to n.m.r. analysis (see below).

Approx. 20 μmol of the InsP_5 sample was dissolved in 3 ml of ^2H_2O and neutralized with NaO^2H for use in all n.m.r. experiments. N.m.r. spectra were recorded on a Bruker wide-bore AM400 spectrometer operating at 400 MHz for ^{1}H and 161.9 MHz for ^{31}P; 400 acquisitions were combined for each ^{31}P spectrum. Two-dimensional proton spectra were recorded with a phase-sensitive double quantum filter COSY programme using 512 segments each of 16 scans. The frequencies for decoupling specific ^{31}P resonances were measured from a proton spectrum acquired through the decoupler channel of the ^{31}P probe immediately before decoupling experiments. Clearly this could not be water-suppressed, and one of the proton resonances was hidden within this region. This proton was decoupled by progressively altering the decoupling frequency until there was a change in the ^{31}P spectrum.

Assay of InsP_5 kinase

Assays were of 200–300 μl total volume and contained 0.1 M-KCl, 10 mM-phosphocreatine, 1 mM-dithiothreitol (or 15 mM-2-mercaptoethanol), 5 units of creatine kinase (Sigma)/ml, 5 mM-ATP, 6 mM-$MgCl_2$, 2 mM-EGTA, 25 mM- or 50 mM-Hepes, 0.3 mM-$CaCl_2$ (pH 7.0; 25 °C for *Dictyostelium*-derived and mung-bean-derived kinases, 37 °C for rat-brain-derived kinases). The solution contains approx. 1 mM free Mg^{2+} and 0.1 μM free Ca^{2+}. The InsP_5 substrates used in the assays are defined in the Results section. Assays were quenched with 4.5% $HClO_4$ and 'spiked' with phytate hydrolysate (10 μl of a solution containing 1.2 mg of total phosphorus/ml) and 500–1000 d.p.m. of [^{32}P]InsP_6. The acid-precipitated protein was pelleted by centrifugation, and the supernatants were removed and mixed with 2 M-KOH/0.1 M-Mes/25 mM-EDTA (200 μl). The final pH of the samples was 6.3–6.8. The $KClO_4$ was sedimented by

centrifugation and the supernatants were applied either to an anion-exchange h.p.l.c. column or to batch-eluted resin columns (see above).

Removal of scintillation fluid and/or h.p.l.c. eluates from inositol phosphate preparations

Inositol phosphates that had been eluted from h.p.l.c. columns in phosphate buffers were desalted as described by Stephens *et al.* (1988*b*). In some situations, inositol phosphates were extracted from scintillation fluid (as described in Stephens & Downes, 1990).

Periodate oxidation, reduction and dephosphorylation of inositol phosphates

H.p.l.c.-purified desalted preparations of inositol phosphates were periodate-oxidized, reduced and dephosphorylated precisely as described by Stephens (1990) and Stephens *et al.* (1988*a*). Unknown InsP_4s were oxidized with 0.1 M-periodic acid (pH 2.0 with NaOH): under these conditions D-Ins(1,2,3,4)P_4 and L-Ins(1,2,3,4)P_4 give L-altritol and D-altritol respectively, and D-Ins(1,2,5,6)P_4 and L-Ins(1,2,5,6)P_4 give D-glucitol and L-glucitol respectively. All periodate oxidations were for 36 h in the dark at 20–25 °C. InsP_3s were oxidized with 0.1 M-sodium periodate. To prepare periodate-sensitive InsP_3s and InsP_4s from cell-derived D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5, the D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5 was partially dephosphorylated with *Aspergillus* phytase. Conditions were exactly as described above for InsP_6, except that the phosphatase was used at 0.0016 unit/ml.

Oxidation of arabitol and glucitol by L-iditol dehydrogenase

L-Arabitol (but not D) and D-glucitol (but not L) are oxidized by L-iditol dehydrogenase (see Fig. 2 and Stephens, 1990). Samples of [^{3}H]arabitol of unknown configuration were incubated with L-iditol dehydrogenase (9.5 units/ml) and D-[^{14}C]arabitol {prepared from D-[^{14}C]arabinose (ICN Radiochemicals) as described by Stephens *et al.* (1988*a*)} exactly as described previously (Stephens *et al.*, 1989).

L- and D-arabitol were prepared by reduction of L- and D-arabinose respectively as described previously (Stephens *et al.*, 1988*a*). L-[^{3}H]Arabitol was prepared from L-arabinose by reduction with NaB^3H_4; 2 μg of L-arabinose (0.133 μmol) was mixed with NaB^3H_4 (1.33 μmol, 0.6 mCi in 7.5 μl total volume). After 8 h the sample was mixed with 1 ml of Bio-Rad AG50 (H^+ form) and processed as described previously (Stephens *et al.*, 1988*a*); 6 μCi of L-[^{3}H]arabitol was finally recovered.

Samples of [^{3}H]glucitols were incubated with L-iditol dehydrogenase (2.5 units/ml) and D-[^{14}C]glucitol (Amersham) as described previously (Stephens *et al.*, 1989). Polyols were separated on cation-exchange columns in the Pb^{2+} form (as described previously; Stephens *et al.*, 1989).

RESULTS

Definition and resolution of InsP_5 standards

To enable the InsP_5s in different tissues to be identified, a convenient chromatographic protocol and an appropriate set of 'standards' were required. There are six InsP_5 isomers, which theoretically divide into four chromatographically distinct groups: Ins(1,3,4,5,6)P_5, Ins(1,2,3,4,6)P_5 (both of which contain a plane of symmetry) and D- and L-Ins(1,2,3,4,5)P_5 and D- and L-Ins(1,2,4,5,6)P_5 (see paragraph on nomenclature in the Introduction).

Ins(1,3,4,5,6)P_5 is the InsP_5 found at a high concentration in avian erythrocytes (Johnson & Tate, 1969) and can be readily prepared labelled with either ^{32}P or ^{3}H for use as a standard (Stephens *et al.*, 1988*a*,*b*).

D- and/or L-Ins(1,2,4,5,6)P_5 was established to be the major InsP_5 in germinating mung beans (in terms of both phosphate content and, in [^{32}P]P_i- or [^{3}H]-Ins-prelabelled cells, ^{32}P or ^{3}H content). An acid extract from 25 g (dry wt.) of mung beans was prepared, mixed with a portion of ^{32}P-labelled inositol phosphates extracted from a [^{32}P]P_i-labelled germinating mung bean, and the major ^{32}P-labelled InsP_5 was purified to homogeneity by anion-exchange chromatography (see Fig. 3); 20.6 μmol of an InsP_5 was recovered and submitted to n.m.r. analysis (see the Materials and methods section, and Fig. 4 and its legend). The results established that the major InsP_5 in germinating mung beans is D- and/or L-Ins(1,2,4,5,6)P_5. This source of ^{3}H- or ^{32}P-labelled D- and/or L-Ins(1,2,4,5,6)P_5 was then used, by co-chromatography (see below), to confirm that: (*a*) the principal InsP_5 formed by a commercially available fungal phytase (from *Aspergillus*; see the Materials and methods section) was also D- and/or L-Ins(1,2,4,5,6)P_5 (as described previously; Irving & Cosgrove, 1972) and (*b*) the predominant InsP_5 generated during alkaline hydrolysis of InsP_6 was D/L-Ins(1,2,4,5,6)P_5 (Cosgrove, 1980).

D- and/or L-Ins(1,2,3,4,5)P_5 [predominantly L-; see Tomlinson & Ballou (1961) and later Lim & Tate (1973)] was prepared from InsP_6 with a commercially available wheat-bran phytase (see the Materials and methods section). Once the wheat-bran phytase-derived InsP_5 was available, it enabled us to establish that the only other detectable InsP_5 found in germinating mung beans was D- and/or L-Ins(1,2,3,4,5)P_5 (results not shown).

With three of the four possible chromatographic classes of InsP_5 (prepared as described above) to serve as markers, Ins(1,2,3,4,6)P_5 was then identified in (and subsequently isolated from) alkaline hydrolysates of InsP_6 by a process of elimination.

The process of characterizing various sources of InsP_5s not only represented a mechanism to verify the identity of those InsP_5

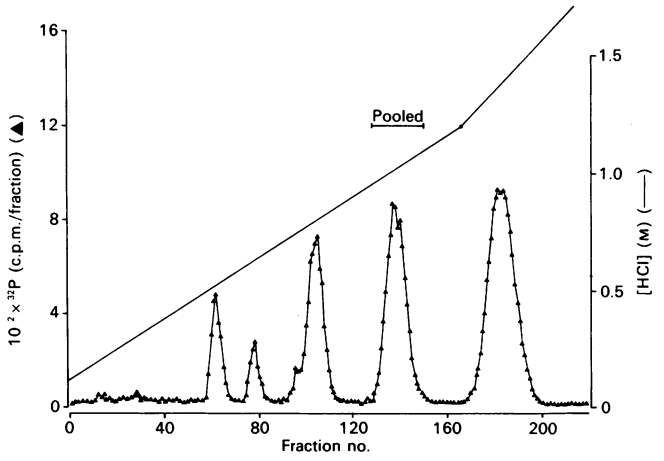

Fig. 3. Purification of mung-bean InsP_5s by anion-exchange chromatography

An acid extract from 25 g/dry wt. of germinating mung beans was prepared as described in the Materials and methods section and mixed with [^{32}P]InsP_4s (two), [^{32}P]InsP_5s (two) and [^{32}P]InsP_6 (h.p.l.c. purified from a [^{32}P]P_i-labelled mung-bean preparation) and loaded on to a 62 cm × 2 cm column of Bio-Rad AG 1 × 8 200–400 resin (chloride form). The column was eluted with HCl at 2.5 ml/min as shown in the Figure and as described in the Materials and methods section (24 ml fractions were collected). The second eluted [^{32}P]InsP_5 (marked in the Figure) was collected and desalted; 20.6 μmol of InsP_5 was finally recovered and submitted for n.m.r. analysis (see Fig. 4).

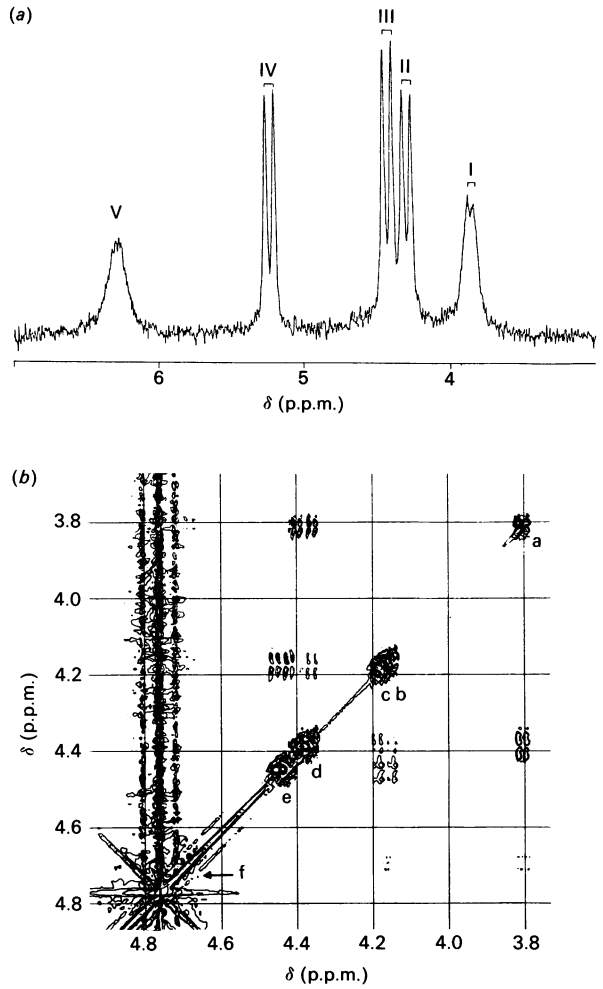

Fig. 4. N.m.r. analysis of mung-bean-derived InsP_5

(*a*) ^{31}P spectrum of the mung-bean InsP_5 (operation at 161.9 MHz); (*b*) ^{1}H COSY spectrum of the mung-bean InsP_5 (operation at 161.9 MHz). The ^{31}P spectrum of the unknown compound showed five peaks labelled I–V in (*a*). ^{31}P signals originating from the phosphates attached to C-1 and C-3, or C-4 and C-6, would be expected to be magnetically very similar (Zuiderweg *et al.*, 1979), so that the presence of only one closely spaced pair of resonances suggested that the non-phosphorylated carbon is one of these four. A double quantum filtered COSY proton spectrum (*b*) indicated one proton resonance shifted downfield enough to be hidden within the residual water resonance. This can only be the anomeric proton attached to a phosphorylated C-2 (Radenberg *et al.*, 1989) and is labelled 'f' in (*b*). Comparison with spectra from Radenberg *et al.* (1989), Mayr & Dietrich (1987) and Cerdan *et al.* (1986), as well as examination of connectivity from the COSY spectrum allocates resonances 'e' and 'd' to protons from C-4 and C-6 (order not determined), resonances 'c' and 'b' to protons from C-5 and C-1 (or C-3) (order again not determined) and resonance 'a' to the proton from C-1 or C-3. The response of the ^{31}P spectrum to narrow-band proton-decoupling linked proton resonances 'b', 'c', 'd' and 'e' to phosphorus resonances IV, I, II and III respectively, i.e. C-4, C-5 and C-6, are phosphorylated and one of C-1 or C-3. Progressive decoupling within narrow frequency ranges in the region of the proton resonance 'f' (see the Materials and methods section) confirmed that this was linked to phosphorus V, i.e. C-2 is phosphorylated. Hence the unknown inositol compound is D- and/or L-Ins(1,2,4,5,6)P_5.

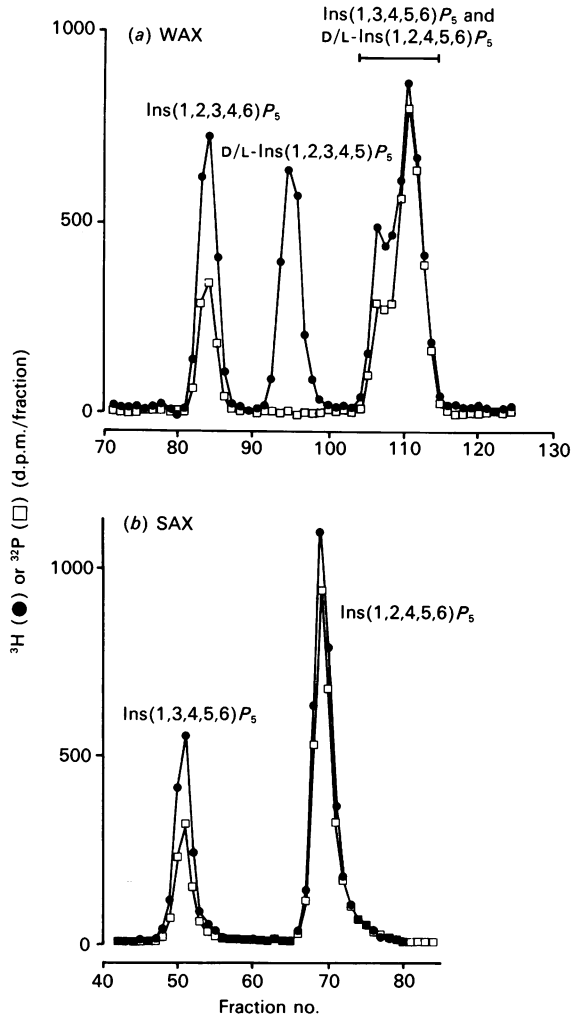

Fig. 5. Resolution by h.p.l.c. of [^{32}P]InsP_5s in an acid extract from [^{32}P]P_i-prelabelled *Dictyostelium* amoebae

NC4 amoebae were incubated with [^{32}P]P_i for 4 h and an acid extract was prepared and mixed with various [^{3}H]InsP_5s {[^{3}H]Ins(1,3,4,5,6)P_5, D/L-[^{3}H]Ins(1,2,4,5,6)P_5, [^{3}H]Ins(1,2,3,4,6)P_5 and D/L-[^{3}H]Ins(1,2,3,4,5)P_5; see the Materials and methods section}. The sample was then chromatographed on a Partisphere WAX h.p.l.c. column. The region in which the [^{3}H]InsP_5s were eluted is shown in (*a*). They were eluted in the order [^{3}H]Ins(1,2,3,4,6)P_5, D/L-[^{3}H]Ins(1,2,3,4,5)P_5, [^{3}H]Ins(1,3,4,5,6)P_5 and D/L-[^{3}H]Ins-(1,2,4,5,6)P_5 (increasing retention time). The peak marked with a horizontal bar was pooled, desalted and chromatographed on a Partisphere SAX h.p.l.c. column (40% of each fraction was removed and counted for ^{3}H and ^{32}P radioactivity). The region in which the [^{3}H]InsP_5s were eluted is shown in (*b*). The [^{3}H]- and [^{32}P]-InsP_5s were resolved into two peaks, the first-eluted being [^{3}H]Ins(1,3,4,5,6)P_5 and the second D/L-[^{3}H]Ins(1,2,4,5,6)P_5.

isomers, but also enabled both ^{3}H- and ^{32}P-labelled forms of all the chromatographically distinct classes of InsP_5 to be prepared for use as standards.

The chromatographic conditions that evolved in parallel with the process of preparing and testing the InsP_5 preparations described above are based on those standardly used during h.p.l.c. analysis of InsP_3s and InsP_4s (see the Materials and methods section). On a weak anion-exchange h.p.l.c. column the order of elution was Ins(1,2,3,4,6)P_5, Ins(1,2,3,4,5)P_5, Ins(1,3,4,5,6)P_5 and Ins(1,2,4,5,6)P_5 (in order of increasing retention time, the last two isomers running relatively close together); on a strong anion-exchange h.p.l.c. column, the order was Ins(1,3,4,5,6)P_5, Ins(1,2,3,4,5)P_5, Ins(1,2,3,4,6)P_5 and Ins(1,2,4,5,6)P_5 (the last two isomers are relatively close together). This compares with 60 cm-long AG-1 (Cl^- form) anion-exchange columns eluted with HCl, in which the elution order is Ins(1,2,3,4,6)P_5, Ins(1,2,3,4,5)P_5, Ins(1,2,4,5,6)P_5 and

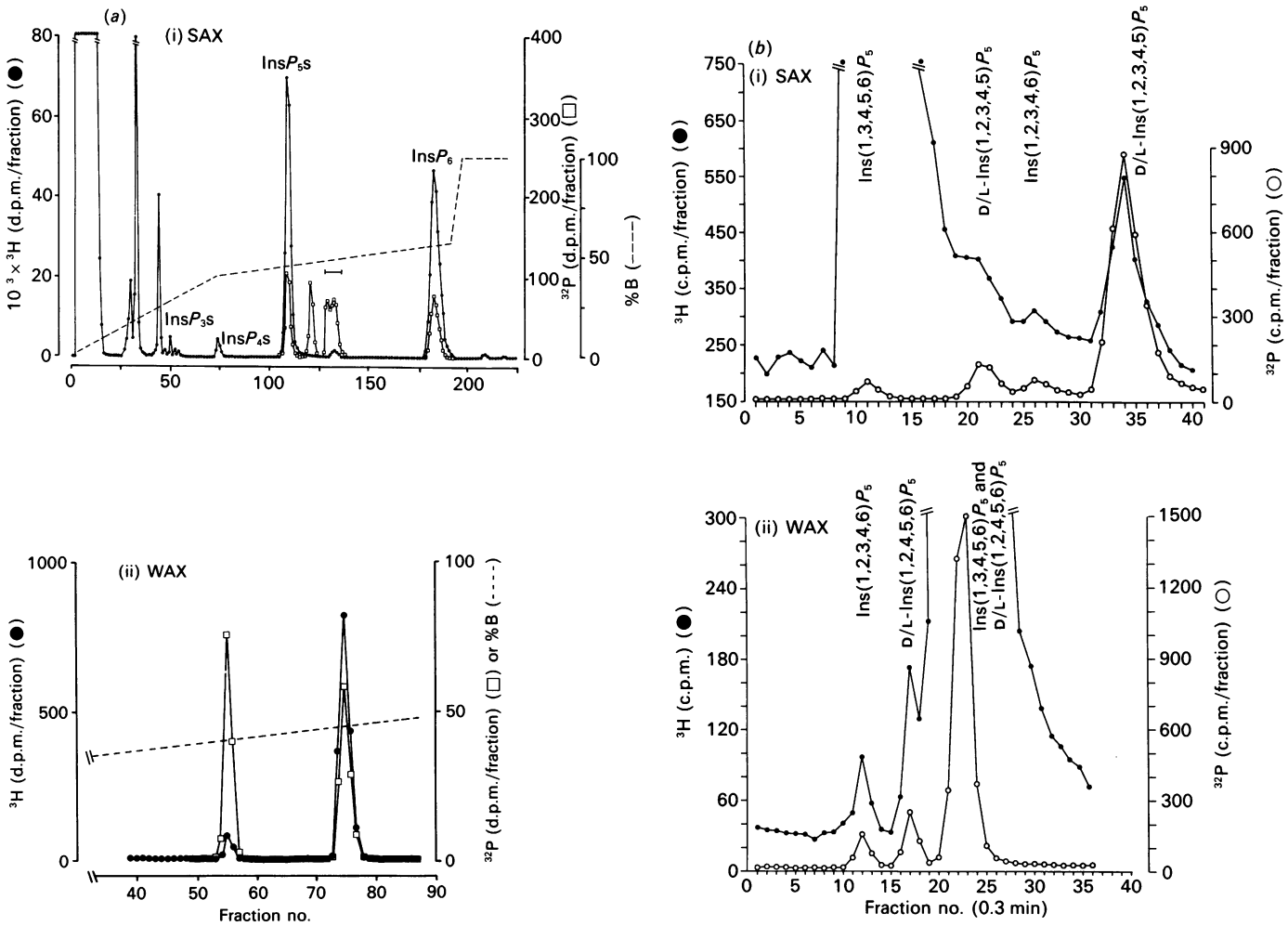

Fig. 6. Resolution of [^{3}H]InsP_5s in [^{3}H]Ins-prelabelled HL60 cells and NG-115-401L-C3 cells

(*a*) HL60 cells were prelabelled with [^{3}H]Ins and an acid extract was prepared and mixed with [^{32}P]InsP_6, [^{32}P]Ins(1,3,4,5,6)P_5, [^{32}P]Ins(1,2,3,4,6)P_5, D/L-[^{32}P]Ins(1,2,3,4,5)P_5 and D/L-[^{32}P]Ins(1,2,4,5,6)P_5 (see the Materials and methods section). The sample was filtered, applied to a Partisphere SAX h.p.l.c. column, eluted and counted (50% of each fraction) for ^{3}H and ^{32}P radioactivity (*ai*). The standards were eluted in the order [^{32}P]Ins(1,3,4,5,6)P_5, D/L-[^{32}P]Ins(1,2,3,4,5)P_5, [^{32}PIns(1,2,3,4,6)P_5 and D/L-[^{32}P]Ins(1,2,4,5,6)P_5 (the last two together, in order of increasing retention time). The fractions marked by a horizontal bar were pooled, desalted and re-applied to a Partisphere WAX h.p.l.c. column. The region in which the InsP_5s were eluted is shown (*aii*). The standards were eluted in the order [^{32}P]Ins(1,2,3,4,6)P_5 and D/L-[^{32}P]Ins(1,2,4,5,6)P_5 (in order of increasing retention time). [^{3}H]Ins-prelabelled HL60 cells contained [^{3}H]Ins(1,3,4,5,6)P_5 (in substantial excess over the other isomers), [^{3}H]Ins(1,2,3,4,6)P_5 and D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5. (*b*) NG-115-401L-C3 cells were prelabelled with [^{3}H]Ins and an acid extract was prepared and mixed with [^{32}P]Ins(1,3,4,5,6)P_5, [^{32}P]Ins(1,2,3,4,6)P_5, D/L-[^{32}P]Ins(1,2,3,4,5)P_5 and D/L-[^{32}P]Ins(1,2,4,5,6)P_5 (see the Materials and methods section). The sample was filtered, divided into two, and one half was chromatographed on a Partisphere SAX h.p.l.c. column (*bi*) and the other on a Partisphere WAX h.p.l.c. column (*bii*). The regions in which the InsP_5s were eluted are shown. Approx. 91000 c.p.m. and 65000 c.p.m. of [^{3}H]Ins(1,3,4,5,6)P_5 was recovered from the SAX and WAX columns respectively. The order of elution of the [^{32}P]InsP_5 standards is described in the legends to Figs. 5 and 6*a* (above). [^{3}H]Ins-prelabelled NG-115-401L-C3 cells contained [^{3}H]Ins(1,3,4,5,6)P_5 (in substantial excess over the other isomers), [^{3}H]Ins(1,2,3,4,6)P_5, D- and/or L-Ins(1,2,3,4,5)P_6 and D- and/or L-Ins(1,2,4,5,6)P_5. This experiment has been repeated once with very similar results.

Ins(1,3,4,5,6)P_5 (Cosgrove, 1969; P. T. Hawkins & L. R. Stephens, results not shown). In all cases the order of elution was established by co-injection of different combinations of various contrastingly labelled (^{32}P or ^{3}H) InsP_5s.

InsP_5s in germinating mung beans

The presence of D- and/or L-Ins(1,2,4,5,6)P_5 and D- and/or L-Ins(1,2,3,4,5)P_5 in germinating mung beans was established as part of the process of preparing 'standard' InsP_5s and characterizing methods capable of separating them as described above. In [^{3}H]Ins- or [^{32}P]P_i-labelled preparations less than 4% of the total radiolabelled InsP_5 fractions were in Ins(1,3,4,5,6)P_5 and Ins(1,2,3,4,6)P_5. During the germination of many seeds there is a net breakdown of the InsP_6 deposited in the storage tissues (Williams, 1970). However, because both [^{3}H]Ins and [^{32}P]P_i are rapidly incorporated into InsP_6 during the germination of the mung-bean seed (see above; Stephens, 1990; Biswas & Biswas, 1965), it can be reasoned that significant synthesis of InsP_6 is also occurring, presumably in a different tissue. Clearly the presence of particular InsP_5 species during germination could be associated with the process of either synthesis or degradation of InsP_6.

InsP_5 in *Dictyostelium*

Acid extracts of [^{32}P]P_i-prelabelled *Dictyostelium discoidium* amoebae were prepared, spiked with [^{3}H]InsP_5 standards, and resolved sequentially on weak and strong anion-exchange h.p.l.c. columns (see Fig. 5 and the Materials and methods section). ^{32}P-labelled compounds co-migrated on a weak anion-exchange

Table 1. Dephosphorylation of *Dictyostelium*-derived D-[^{3}H]Ins(1,2,4,5,6)P_5 and identification of the products

The D-[^{3}H]Ins(1,2,4,5,6)P_5 preparations were purified from [^{3}H]Ins-prelabelled amoebae and then dephosphorylated with an *Aspergillus* phytase preparation (see the Materials and methods section). The [^{3}H]InsP_4 and [^{3}H]InsP_3 products were purified by h.p.l.c., then periodate-oxidized, reduced and dephosphorylated (see the Materials and methods section). The [^{3}H]polyols recovered were identified and incubated with L-iditol dehydrogenase (see the Materials and methods section).

D-[^{3}H]Ins(1,2,4,5,6)P_5 preparation (initial d.p.m.)	Percentage of total [^{3}H]InsP_5 dephosphorylated	Percentage of total ^{3}H in [^{3}H]InsP_4 or [^{3}H]InsP_3 to be analysed	Polyols recovered (% recovery)	Oxidation with L-iditol dehydrogenase (overall % recovery)
A (28000 d.p.m.)	50%	8% [^{3}H]InsP_3(s)	[^{3}H]Arabitol (70%)	D-[^{3}H]Arabitol (80%)
B (90000 d.p.m.)	28%	10% [^{3}H]InsP_4(s)	70% [^{3}H]glucitol 30% [^{3}H]Ins (85%)	89% D-glucitol 11% L-glucitol (90%)

column with [^{3}H]Ins(1,2,3,4,6)P_5 and the peak containing D/L-[^{3}H]Ins(1,2,4,5,6)P_5 and [^{3}H]Ins(1,3,4,5,6)P_5. When the latter peak was desalted and re-applied to a strong anion-exchange h.p.l.c. column, both the ^{3}H and ^{32}P were resolved into two peaks. Furthermore, amoebae which were prelabelled with [^{3}H]Ins yielded acid extracts containing [^{3}H]Ins-labelled peaks which co-migrated with [^{32}P]Ins(1,2,4,5,6)P_5, [^{32}P]Ins(1,2,3,4,6)P_5 and [^{32}P]Ins(1,3,4,5,6)P_5 (results not shown). Together, these observations suggest that NC4 amoebae contain at least three InsP_5s: Ins(1,2,3,4,6)P_5, Ins(1,3,4,5,6)P_5 and D- and/or L-Ins(1,2,4,5,6)P_5. InsP_5s and InsP_6 were purified from [^{3}H]Ins-prelabelled amoebae, and both their intracellular radiochemical concentrations (in d.p.m./μl) and the specific radioactivity of their inositol moieties were determined (by dephosphorylating the inositol polyphosphates with alkaline phosphatase and assaying both the concentration and ^{3}H content of the liberated inositol; see Stephens & Irvine, 1990), and hence their original intracellular chemical concentrations could be calculated. Ins(1,3,4,5,6)P_5, Ins(1,2,3,4,6)P_5, D- and/or L-Ins(1,2,4,5,6)P_5 and InsP_6 were present at concentrations of 8 μM, 16 μM, 36 μM and 600 μM respectively (Stephens & Irvine, 1990).

InsP_5s in mammalian cell lines

[^{3}H]Ins-prelabelled HL60 and NG115 401L-C3 cells yielded acid extracts containing relatively very large amounts of [^{3}H]Ins(1,3,4,5,6)P_5 and smaller amounts of D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5, D- and/or L-[^{3}H]Ins(1,2,3,4,5)P_5 and [^{3}H]Ins(1,2,3,4,6)P_5 (see Fig. 6). It is unlikely that the radioactivity in the D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5 peak results from acid-catalysed phosphate migration of [^{3}H]Ins(1,3,4,5,6)P_5, which would cause D/L-Ins(1,2,4,5,6)P_5 formation, as similar extraction protocols applied to avian erythrocytes have reproducibly given acid extracts containing no detectable D/L-Ins(1,2,4,5,6)P_5 (results not shown). Identical relative distributions of [^{3}H]Ins label between these [^{3}H]InsP_5s and [^{3}H]InsP_6 were seen after 10 days labelling with [^{3}H]Ins under these conditions; over 99% of the cells in the cultures had been 'synthesized' in the presence of [^{3}H]Ins, hence it is very likely this distribution of ^{3}H radioactivity is a true reflection of the inositol phosphates' relative concentrations.

Stereochemical assignment of D- and/or L-Ins(1,2,4,5,6)P_5 found in *Dictyostelium discoidium* amoebae

D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5 was h.p.l.c.-purified (by both strong and weak anion-exchange h.p.l.c. columns) from [^{3}H]Ins-prelabelled amoebae, then partially dephosphorylated with an *Aspergillus*-derived phytase preparation (see the Materials and methods section). Two independent preparations of D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5 were dephosphorylated to different extents (see Table 1) and the products were resolved by anion-exchange h.p.l.c. The [^{3}H]InsP_3 peak yielded [^{3}H]arabitol and was therefore either D- or L-[^{3}H]Ins(1,2,6)P_3; traces of [^{3}H]glucitol were also obtained, but because of the ambiguity in the way [^{3}H]glucitol can be derived from [^{3}H]InsP_3s this was not further analysed. The [^{3}H]InsP_4 peak yielded [^{3}H]glucitol, meaning some D- or L-[^{3}H]Ins(1,2,5,6)P_4 was present. Also formed (Table 1) was [^{3}H]Ins, which could be derived from D- or L-[^{3}H]Ins(1,2,4,5)P_4, D- or L-[^{3}H]Ins(1,2,4,6)P_4 or [^{3}H]Ins(2,4,5,6)P_4 [if it is accepted that only InsP_4 derived from D- and/or L-Ins(1,2,4,5,6)P_5 could not possess phosphate groups at both the D- and L-3 positions]. The alternative explanation for the presence of [^{3}H]Ins amongst the periodate-oxidation, reduction and dephosphorylation products of the [^{3}H]InsP_4(s) is that the [^{3}H]InsP_4 that yielded [^{3}H]glucitol was incompletely oxidized; this is unlikely to be true, as independent preparations of D-[^{3}H]Ins(1,2,5,6)P_4 that were oxidized in parallel only gave D-[^{3}H]glucitol (see Stephens, 1990; L. R. Stephens, unpublished work). D- but not L-glucitol is oxidized to fructose by L-iditol dehydrogenase (Stephens *et al.*, 1990; Stephens, 1990). L- but not D-arabitol is oxidized to ribulose and xylulose by L-iditol dehydrogenase (see Fig. 2, the Materials and methods section and Stephens, 1990). The substrate selectivity of yeast-derived L-iditol dehydrogenase was therefore exploited to determine the chirality of the [^{3}H]arabitol and [^{3}H]glucitol which had originated from the dephosphorylation products of D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5. The assays were designed to operate according to the principles previously defined (Stephens *et al.*, 1988*a*, 1989; Stephens, 1990; see the legend to Fig. 2). The [^{3}H]arabitol in question was completely unoxidized (88% of the starting material was recovered) in an assay in which 50% of the starting internal L-arabitol had been oxidized. Some 80% of the [^{3}H]glucitol was oxidized in an assay in which 90% of the internal D-[^{14}C]glucitol had been oxidized. These results are consistent with the [^{3}H]InsP_3 and [^{3}H]InsP_4 derived from the D- and/or L-Ins(1,2,4,5,6)P_5 possessing the structures D-[^{3}H]Ins(1,2,6)P_3 and D-[^{3}H]Ins(1,2,5,6)P_4 and hence the original ^{3}H-InsP_5 must have been D-[^{3}H]Ins(1,2,4,5,6)P_5. The small quantity of L-[^{3}H]glucitol detected in the latter assay (approx. 11% of the total) suggests that in that preparation 11% of the [^{3}H]InsP_4 was L-[^{3}H]Ins(1,2,5,6)P_4, and hence some of the original [^{3}H]InsP_5 was possibly L-[^{3}H]Ins(1,2,4,5,6)P_5. However, the exact proportions of D- or L-[^{3}H]Ins(1,2,4,5,6)P_5 in the original extract cannot be assessed, as the stereoselectivity of the *Aspergillus* phytase for, and hence the relative rates of attack on, D- or L-Ins(1,2,4,5,6)P_5 are unknown (it is unlikely to be highly

Table 2. Subcellular distribution of D-[^{3}H]Ins(1,2,4,5,6)P_5 hydroxy kinase in *Dictyostelium*

A suspension of *Dictyostelium* was homogenized by filtration. A soluble fraction was prepared from this by centrifugation (100000 *g*, 90 min, 4 °C) and the pellet was resuspended in lysis buffer to the volume of the original filtrate to yield a particulate fraction. Triton X-114 was added to portions of each of the fractions (final concn. 0.2%, w/v), and samples of all the fractions were diluted 200-fold into 25 mM-Hepes/1 mM-EGTA/1 mM-dithiothreitol/1 mg of BSA/ml (pH 7.0, 4 °C) and assayed for [^{3}H]Ins(1,2,4,5,6)P_5 hydroxy kinase activity as described in the Materials and methods section. The assays each contained 20000 d.p.m. of D/L-[^{3}H]Ins(1,2,4,5,6)P_5. The data are means ($n = 2$) from a single experiment which was representative of two other similar experiments.

Treatment	% of total ^{3}H in assay in ^{3}H-InsP_6
No enzyme	0
Filtrate	12.6
Filtrate + X-114	12.8
Particulate	1.0
Particulate + X-114	0.8
Soluble	11.5
Soluble + X-114	10.9

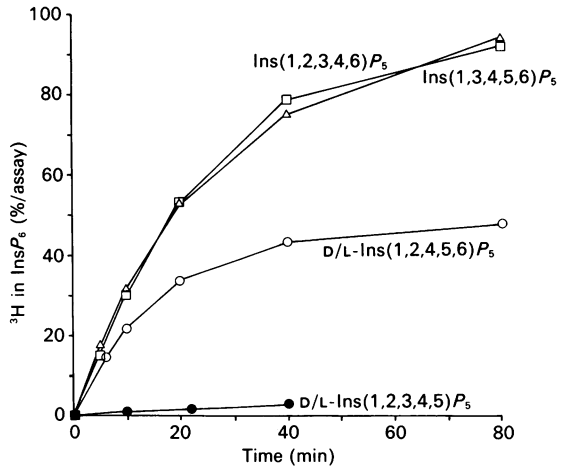

Fig. 7. Phosphorylation of [^{3}H]InsP_5s in slime-mould cytosolic fractions

A cytosolic fraction was prepared from *Dictyostelium* NC4 amoebae and diluted to various extents into 25 mM-Hepes/1 mM-EGTA/1 mM-dithiothreitol/1 mg of BSA/ml (pH 7.0, 4 °C). Samples (150 μl) of the appropriate dilutions of the crude cytosol fraction were incubated with standard [^{3}H]InsP_5s (all approx. 60 Ci/mmol) for various times (in assays of 200 μl total volume). D/L-[^{3}H]Ins(1,2,4,5,6)P_5 (○; 90000 d.p.m./assay) was incubated with a 200-fold dilution of crude cytosol; [^{3}H]Ins(1,3,4,5,6)P_5 (△; 50000 d.p.m./assay) was incubated with a 10-fold dilution of crude cytosol; [^{3}H]Ins(1,2,3,4,6)P_5 (□; 45000 d.p.m./assay) was incubated with a 40-fold dilution of crude cytosol; and D/L-[^{3}H]Ins(1,2,3,4,5)P_5 (●; 100000 d.p.m./assay) was incubated with undiluted crude cytosol. The assay constituents and sample analysis were as described in the Materials and methods section. The data are presented as the mean ($n = 2$) percentage of the total ^{3}H (d.p.m.) in the assay that was recovered as [^{3}H]InsP_6. The results are typical of three similar experiments.

selective, as it is capable of dephosphorylating at least three other [^{3}H]InsP_5s as well as [^{3}H]InsP_6; L. R. Stephens, unpublished work). This was the only evidence that we obtained that was consistent with the presence of any L-[^{3}H]Ins(1,2,4,5,6)P_5 in NC4 amoebae, and furthermore numerous independent preparations

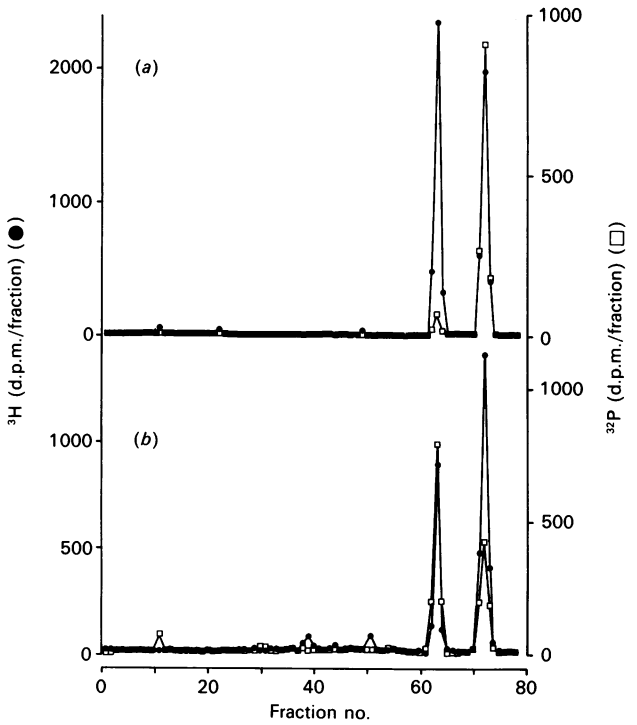

Fig. 8. Substrate specificity of a *Dictyostelium* Ins(1,2,4,5,6)P_5 hydroxy kinase activity

A suspension of NC4 amoebae was permeabilized by electroporation and incubated for 0 min (results not shown) or 40 min with mixtures of either (*a*) [^{32}P]Ins(1,2,4,5,6)P_5 (□, D- and/or L; h.p.l.c.-purified from [^{32}P]P_i-labelled NC4 amoebae) and D/L-[^{3}H]Ins(1,2,4,5,6)P_5 (●, racemic), or (*b*) [^{3}H]Ins(1,2,4,5,6)P_5 (●; derived from [^{3}H]InsP_6 through the action of an *Aspergillus* phytase) and D/L-[^{32}P]Ins(1,2,4,5,6)P_5 (□, racemic): see the Materials and methods section for details. The assays were quenched, processed and chromatographed on a Partisphere SAX h.p.l.c. column as described in the Materials and methods section. The eluate was collected into fractions which were counted for ^{3}H and ^{32}P radioactivity as described. The results are typical of those obtained with two independent preparations of [^{32}P]Ins(1,2,4,5,6)P_5 (from amoebae) and three preparations of [^{3}H]Ins(1,2,4,5,6)P_5 (produced by the *Aspergillus* phytase). The zero-time incubations all contained a single peak of ^{3}H and ^{32}P radioactivity which was eluted at the time expected for D/L-Ins(1,2,4,5,6)P_5.

of D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5 from [^{3}H]Ins-prelabelled amoebae appeared completely homogeneous when assayed with a kinase that is totally selective for D-Ins(1,2,4,5,6)P_5 (see below).

InsP_5 kinase activities in amoebae

When [^{3}H]Ins(1,3,4,5,6)P_5, [^{3}H]Ins(1,2,3,4,6)P_5, D/L-[^{3}H]Ins-(1,2,4,5,6)P_5 and D/L-[^{3}H]Ins(1,2,3,4,5)P_5 (all 60 Ci/mmol) were incubated with amoebae homogenates in the presence of ATP, then [^{3}H]InsP_6 was formed in assays containing any of the first three substrates. The InsP_5 kinase activities were soluble and showed no significant latency (see Table 2, and results not shown). By diluting cytosolic fractions (into 25 mM-Hepes/1 mM-EGTA/1 mM-dithiothreitol/1 mg of BSA/ml, pH 7.0, 4 °C), it was possible to construct assays which operated under first-order conditions with respect to the quantity of enzyme added. If the progress of these reactions was monitored with time, it was apparent that both [^{3}H]Ins(1,3,4,5,6)P_5 and [^{3}H]Ins(1,2,3,4,6)P_5 could be completely phosphorylated to [^{3}H]InsP_6, whereas a maximum of 49–52% of several independent D/L-[^{3}H]-Ins(1,2,4,5,6)P_5 preparations gave [^{3}H]InsP_6 (less than 5% of

a sample of [^{3}H]InsP_6 was dephosphorylated in any of a set of parallel assays which were run under identical conditions; see Fig. 7). The substrate specificity of the kinase activity which could phosphorylate approx. 50% of a D/L-[^{3}H]Ins(1,2,4,5,6)P_5 preparation was investigated by incubating with amoebae homogenates or cytosolic fractions: (*a*) a mixture of [^{32}P]-D- and/or L-Ins(1,2,4,5,6)P_5 (derived from [^{32}P]P_i-prelabelled amoebae) and racemic D/L-[^{3}H]Ins(1,2,4,5,6)P_5 or (*b*) a mixture of D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5 derived from [^{3}H]InsP_6 through the action of a commercially available *Aspergillus* phytase preparation (see above; its enantiomeric configuration is uncertain) and racemic D/L-Ins[^{32}P](1,2,4,5,6)P_5. The assays were constructed so that the reactions were expected to reach around 50% conversion of the racemic substrates (see Fig. 8). Some 66% of the D- and/or L-[^{3}H]Ins(1,2,4,5,6)P_5 produced by the *Aspergillus* phytase preparation was phosphorylated to [^{3}H]InsP_6 in an assay in which 38% of the total D/L-Ins[^{32}P](1,2,4,5,6)P_5 was phosphorylated to Ins[^{32}P]P_6; extrapolating the extent of reaction to the point where 50% of the total racemic mixture would have been phosphorylated predicts that 87% of the total [^{3}H]Ins-(1,2,4,5,6)P_5 was the D isomer. The slime-mould-derived D- and/or L-Ins[^{32}P](1,2,4,5,6)P_5 was completely converted into Ins[^{32}P]P_6 (see Fig. 8), whereas only 49% of the racemic D/L-[^{3}H]Ins(1,2,4,5,6)P_5 was converted into [^{3}H]InsP_6. These results show that: (i) the slime-mould Ins[^{32}P](1,2,4,5,6)P_5 is homogeneous (i.e. all D or L), (ii) the kinase responsible for its conversion into Ins[^{32}P]P_6 is completely selective for that isomer, and (iii) approx. 8% of the *Aspergillus* phytase product is of the same stereochemical configuration as the slime-mould-derived InsP_5.

On the basis of the data presented in the previous section, we have suggested that the slime-mould-derived Ins(1,2,4,5,6)P_5 is predominantly the D-enantiomer, though we could not rule out some L-isomer being present. The results discussed in the preceding paragraph suggest it is in fact a pure enantiomer, and therefore we conclude that it is D-Ins(1,2,4,5,6)P_5. The data above also show that the fungal phytase product is largely D-Ins(1,2,4,5,6)P_5 as previous data did not establish the precise proportions of the D- and L-enantiomers present, only that there was more of the D-isomer (Irving & Cosgrove, 1972). Hence, altogether these results independently confirm (*a*) that the *Dictyostelium*-derived activity capable of phosphorylating D- or L-Ins(1,2,4,5,6)P_5 is in fact a D-Ins(1,2,4,5,6)P_5 3-hydroxy kinase and (*b*) that the slime-mould-derived [^{3}H]Ins(1,2,4,5,6)P_5 is the D-enantiomer, and furthermore, that 87% of the D- and/or L-Ins(1,2,4,5,6)P_5 that had accumulated in the *Aspergillus*-phytase-catalysed dephosphorylation mixture was of the D-configuration.

The first-order rate constants for the conversion of Ins(1,3,4,5,6)P_5, Ins(1,2,3,4,6)P_5 and D-Ins(1,2,4,5,6)P_5 into InsP_6 in intact amoebae were estimated by extrapolating data from assays with diluted cytosol fractions; they were 0.1, 0.8 and 3.1 s^{-1} respectively [assuming (*a*) a cytosolic protein concentration of 50 mg/ml and (*b*) only half of a racemic mixture of D/L-[^{3}H]Ins(1,2,4,5,6)P_5 represented potential substrate].

InsP_5 kinase activities in rat brain

We also searched for [^{3}H]InsP_5 kinase activities in rat brain extracts. We found soluble, ATP-dependent, heat-labile activities capable of converting all four chromatographic classes of [^{3}H]InsP_5 into [^{3}H]InsP_6. D/L-[^{3}H]Ins(1,2,4,5,6)P_5, D/L-[^{3}H]Ins(1,2,3,4,5)P_5, [^{3}H]Ins(1,2,3,4,6)P_5 and [^{3}H]Ins(1,3,4,5,6)P_5 were converted into [^{3}H]InsP_6 with initial rates, expressed as the percentage of total radioactivity in the assay appearing as [^{3}H]InsP_6, of 1.3, 0.7, 2.7 and approx. 0.05%/min respectively, with a soluble protein concentration of 3.42±0.5 mg/ml in the assays (Fig. 9, and results not shown). The conversion of all

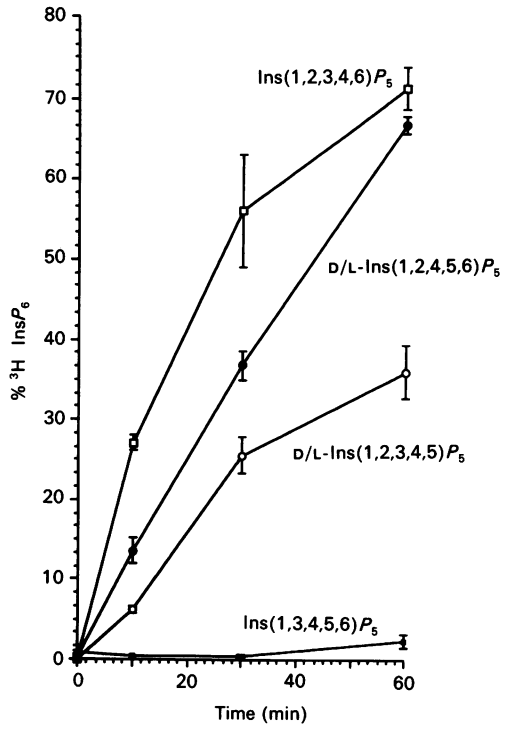

Fig. 9. Phosphorylation of [^{3}H]InsP_5s in rat brain cytosolic fractions

A cytosolic fraction was prepared from rat brain as described in the Materials and methods section (3.57 g wet wt./10.0 ml of buffer). Portions (150 μl) of the undiluted cytosol fraction were incubated with standard [^{3}H]InsP_5s in a total volume of 250 μl for various times (see the Materials and methods section). Approx. 20000 d.p.m. of each [^{3}H]InsP_5 (80 Ci/mmol, made from alkali-catalysed hydrolysis of [^{3}H]InsP_6) was used per assay: [^{3}H]Ins(1,3,4,5,6)P_5 (■), [^{3}H]Ins(1,2,3,4,6)P_5 (□), D/L-[^{3}H]Ins(1,2,4,5,6)P_5 (●) or D/L-[^{3}H]Ins(1,2,3,4,5)P_5 (○). The assay constituents and sample analysis were as described in the Materials and methods section. The data are presented as the mean percentages (±S.D.; $n = 3$) of the total ^{3}H in the assay that was recovered as [^{3}H]InsP_6. The results are typical of three similar experiments (including one in which the [^{3}H]InsP_5 substrates were purified directly from the mixture of [^{3}H]Ins polyphosphates produced during the phosphorylation of [^{3}H]Ins; see the Materials and methods section).

[^{3}H]InsP_5 isomers into [^{3}H]InsP_6 proceeded with no apparent lag and, under the assay conditions employed, [^{3}H]InsP_6 and each of the [^{3}H]InsP_5 isomers were dephosphorylated very slowly; less than 5% of [^{3}H]InsP_6, at the same actual concentration as the [^{3}H]InsP_5 isomers, was dephosphorylated in 120 min (results not shown). We have not yet defined the relative rates of conversion for each enantiomer within each of the two racemic pairs of InsP_5 isomers. It is clear, however, that over 50% conversion of D/L-[^{3}H]Ins(1,2,4,5,6)P_5 into [^{3}H]InsP_6 can be seen with prolonged incubations (Fig. 9), and therefore both D- and L-[^{3}H]Ins(1,2,4,5,6)P_5 must act as substrates for InsP_5 kinase activities in rat brain cytosol. We could detect no InsP_5 hydroxy kinase activities in rat brain particulate fractions, though the rate of dephosphorylation of each [^{3}H]InsP_5 isomer was increased relative to cytosol fractions {D/L-[^{3}H]Ins(1,2,3,4,5)P_5 was the most actively dephosphorylated isomer; results not shown}.

InsP_5 kinase activities in mung bean

[^{3}H]InsP_5 kinase assays were also performed with mung-bean seedling cytosolic fractions. High-speed supernatants prepared from seedling homogenates could phosphorylate all four chromatographic classes of [^{3}H]InsP_5: [^{3}H]Ins(1,3,4,5,6)P_5,

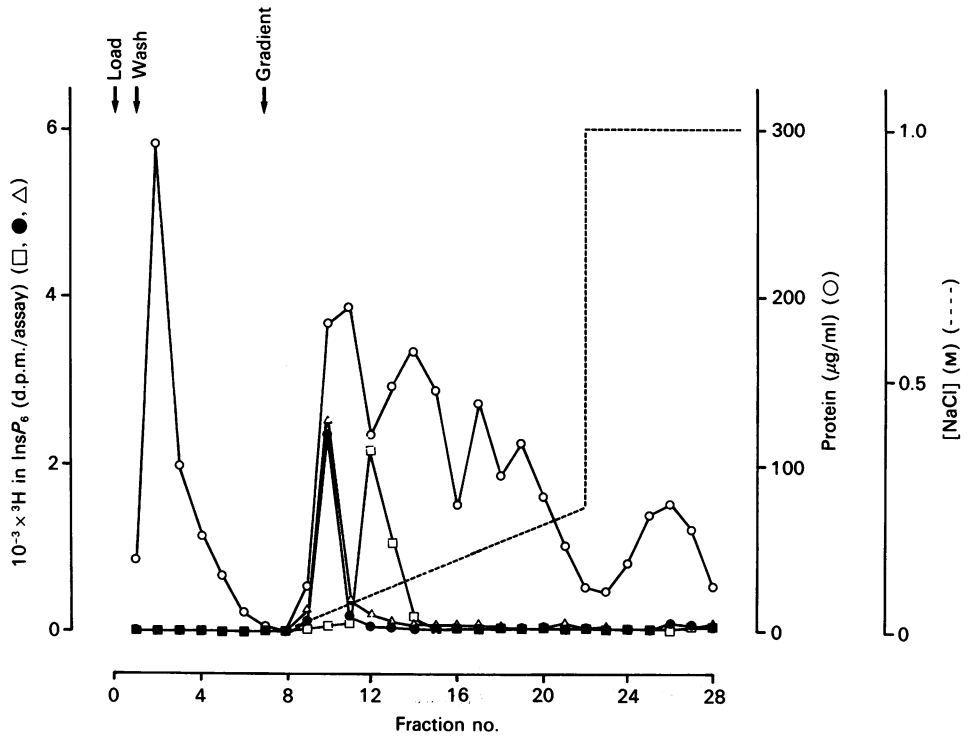

Fig. 10. Resolution of *Dictyostelium* InsP_5 hydroxy kinase activities on DEAE-Sepharose

Dictyostelium cytosolic fraction (4 ml containing 11 mg of total protein; see the Materials and methods section) was loaded on a 4 ml column of DEAE-Sepharose at a flow rate of 1 ml/min. The column was washed with buffer A (25 mM-Hepes/1 mM-EGTA/1 mM-dithiothreitol, pH 7.0, 4 °C) and eluted with a NaCl gradient (in buffer A, as indicated in the Figure; ----). Fractions were collected every 4 min and samples were assayed for protein (○) and for [^{3}H]Ins(1,3,4,5,6)P_5 kinase (□; 100 μl of a 10-fold dilution of each fraction in 200 μl assays), [^{3}H]Ins(1,2,3,4,6)P_5 kinase (●; 100 μl of a 30-fold dilution of each fraction in 200 μl assays), and [^{3}H]Ins(1,2,4,5,6)P_5 kinase (△; 100 μl of a 500-fold dilution of each fraction in 200 μl assays). The assays were run for 30 min, then quenched, mixed with [^{32}P]InsP_6 and resolved into [^{3}H]InsP_5 and [^{3}H]InsP_6 fractions as described in the Materials and methods section. The data shown are typical of three independent separations: 102% of the [^{3}H]Ins(1,3,4,5,6)P_5 kinase, 83% of the D-[^{3}H]Ins(1,2,4,5,6)P_5 kinase and 85% of the [^{3}H]Ins(1,2,3,4,6)P_5 kinase activities that were applied to the column were recovered.

[^{3}H]Ins(1,2,3,4,6)P_5, D/L-[^{3}H]Ins(1,2,4,5,6)P_5 and D/L-[^{3}H]Ins(1,2,3,4,5)P_5 were phosphorylated with first-order rate constants of 0.0066, 0.0024, 0.0002 and 0.0022 s^{-1} respectively, in an undiluted supernatant derived from 9.7 g of wet germinated mung beans homogenized into 10 ml of buffer at a protein concentration of 10 mg/ml (see the Materials and methods section). These rate constants are derived from experiments with supernatant fractions (diluted 50–500-fold into 25 mM-Hepes/1 mM-EGTA/1 mM-dithiothreitol/1 mg of BSA/ml, pH 7.0, at 4 °C; under these conditions the assays were operating under first-order conditions with respect to added mung-bean protein), and then extrapolated to yield estimates of the rates in the original lysate.

The *Dictyostelium* D-Ins(1,2,4,5,6)P_5 hydroxy kinase activity was used to establish the stereochemical configuration of D- and/or L-Ins[^{32}P](1,2,4,5,6)P_5 derived from [^{32}P]P_i-labelled mung-bean seedlings (as above); 79% of the total D- and/or L-Ins[^{32}P](1,2,4,5,6)P_5 was of the L configuration (results not shown).

Resolution of InsP_5 kinase activities in slime moulds

The [^{3}H]InsP_5 kinase activities in slime-mould cytosol fractions could be resolved into two peaks by chromatography on DEAE-Sepharose: [^{3}H]Ins(1,3,4,5,6)P_5 hydroxy kinase was eluted significantly after [^{3}H]Ins(1,2,3,4,6)P_5 and D-[^{3}H]Ins(1,2,4,5,6)P_5 hydroxy kinase activities (see Fig. 10). Furthermore, the effects of a range of inositol polyphosphates on the activity of the three InsP_5 hydroxy kinase activities in crude slime-mould cytosolic fractions allowed at least two functionally independent kinases to be resolved (see Fig. 11). [^{3}H]Ins(1,3,4,5,6)P_5 hydroxy kinase showed a completely different pattern of response compared with [^{3}H]Ins(1,2,3,4,6)P_5 and D-[^{3}H]Ins(1,2,4,5,6)P_5 hydroxy kinases. It should be noted that, in the [^{3}H]Ins(1,3,4,5,6)P_5 hydroxy kinase assays to which small amounts of Ins(1,2,3,4,6)P_5 and D/L-Ins(1,2,4,5,6)P_5 had been added, significant fractions of the cold inositol phosphates would have been converted into InsP_6; similarly large proportions of the lower concentrations of D/L-Ins(1,2,4,5,6)P_5 added to the [^{3}H]Ins(1,2,3,4,6)P_5 hydroxy kinase would have been converted into InsP_6. However, the fact that the inhibition curves deviate substantially from those of InsP_6 (both positively and negatively) shows that this problem is not too severe, and is limited by the low K_m values of these activities for their substrates (see below). The K_m values of the [^{3}H]-Ins(1,2,3,4,6)P_5, [^{3}H]Ins(1,3,4,5,6)P_5 and D-[^{3}H]Ins(1,2,4,5,6)P_5 hydroxy kinase activities in slime-mould cytosolic fractions were determined to be 3.8 μM, 1.6 μM and 1.4 μM respectively (see Fig. 12).

Levels of InsP_6 and the InsP_5 hydroxy kinase activities during differentiation of *Dictyostelium*

Amoebae were harvested from exponentially growing populations of cells and plated out on agar supports in the absence of nutrients to develop into fruiting bodies. Samples of cells were harvested at intervals, and acid extracts were prepared from some (see Fig. 13), whereas others were lysed with detergents and subsequently assayed for all three InsP_5 hydroxy kinase

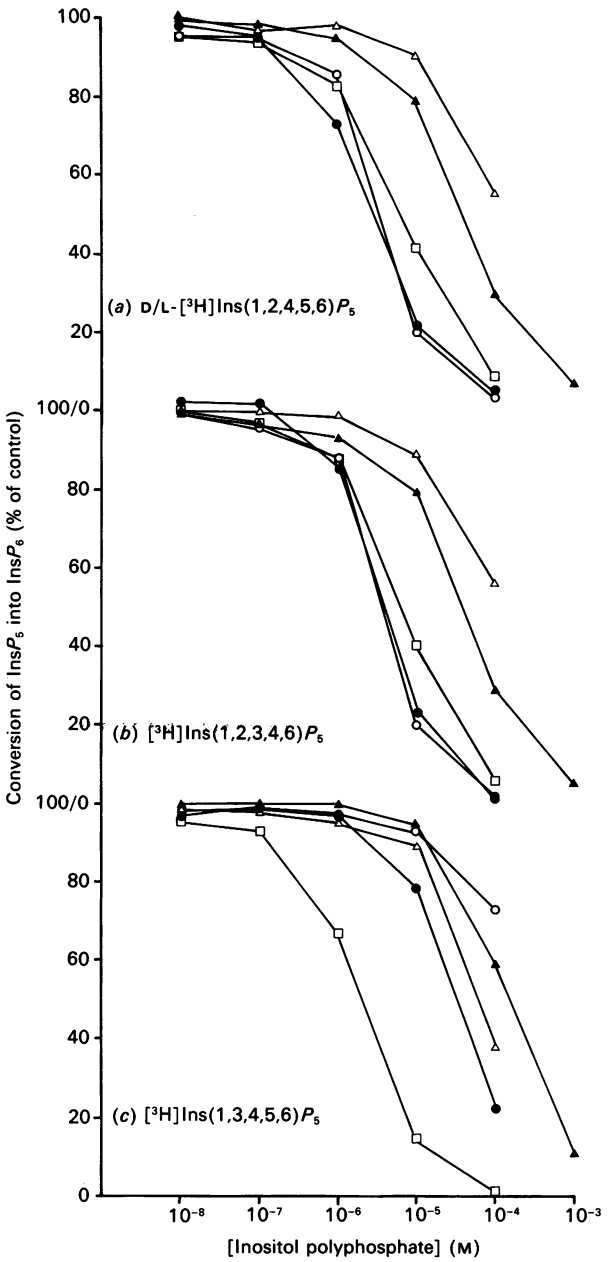

Fig. 11. Differential inhibition of the *Dictyostelium* [^{3}H]InsP_5 hydroxy kinases with inositol polyphosphates

Dictyostelium-derived cytosolic fractions were diluted to various extents and assayed for D-[^{3}H]Ins(1,2,4,5,6)P_5 kinase (*a*), [^{3}H]Ins(1,2,3,4,6)P_5 kinase (*b*) and [^{3}H]Ins(1,3,4,5,6)P_5 kinase (*c*) activities in the presence of a range of concentrations of InsP_6 (▲), 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 (□) and D/L-Ins(1,2,3,4,6)P_5 (○). The assays contained 50000–100000 d.p.m. of substrate, of which a maximum of 22% was converted into [^{3}H]InsP_6 (a maximum of 10% of the total [^{3}H]Ins(1,2,4,5,6)P_5 was converted into [^{3}H]InsP_6). Each point on the graphs is the mean of four determinations, pooled from two independent experiments (the mean S.D. was 3.1%). The data are presented as proportions of the quantity of [^{3}H]InsP_5 converted into [^{3}H]InsP_6 in assays to which no inositol polyphosphates had been added.

activities and the marker enzymes cyclic AMP phosphodiesterase and glycogen phosphorylase (see the Materials and methods section). The concentration of InsP_6 in the acid extracts was determined by phosphorus assay of the fractions eluted from an h.p.l.c. column that contained the ^{32}P from a spike of Ins[^{32}P]P_6

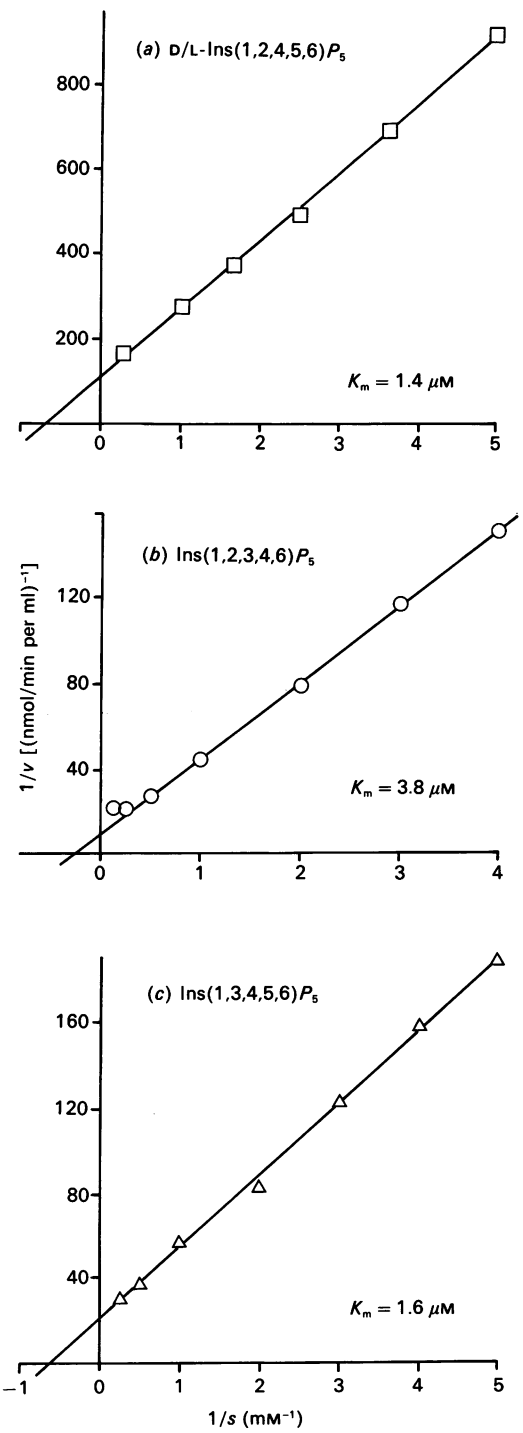

Fig. 12. Determination of the K_m values of the *Dictyostelium* [^{3}H]InsP_5 hydroxy kinases for their InsP_5 substrates

Diluted *Dictyostelium*-derived cytosolic fractions were assayed for D-[^{3}H]Ins(1,2,4,5,6)P_5 kinase (*a*), [^{3}H]Ins(1,2,3,4,6)P_5 kinase (*b*) and [^{3}H]Ins(1,3,4,5,6)P_5 kinase (*c*) as described in the Materials and methods section (and the legend to Fig. 11). A maximum of 20% of the radioactive substrate in any assay was converted into [^{3}H]InsP_6 (a maximum of 9% for the [^{3}H]Ins(1,2,4,5,6)P_5 kinase assay). The data are means (n = 2) derived from single experiments (additional experiments yielded estimates of the K_m that are shown in parentheses below). The apparent K_m of Ins(1,2,4,5,6)P_5 hydroxy kinase for D-Ins(1,2,4,5,6)P_5 (assuming that only half of both the tritiated and unlabelled preparations of D/L-Ins(1,2,4,5,6)P_5 represented potential substrate) was 1.4 μM (1.2 μM); that of [^{3}H]Ins(1,3,4,5,6)P_5 hydroxy kinase for Ins(1,3,4,5,6)P_5 was 1.6 μM (1.7 μM); and that of [^{3}H]Ins(1,2,3,4,6)P_5 hydroxy kinase for Ins(1,2,3,4,6)P_5 was 3.8 μM (3.6 μM).

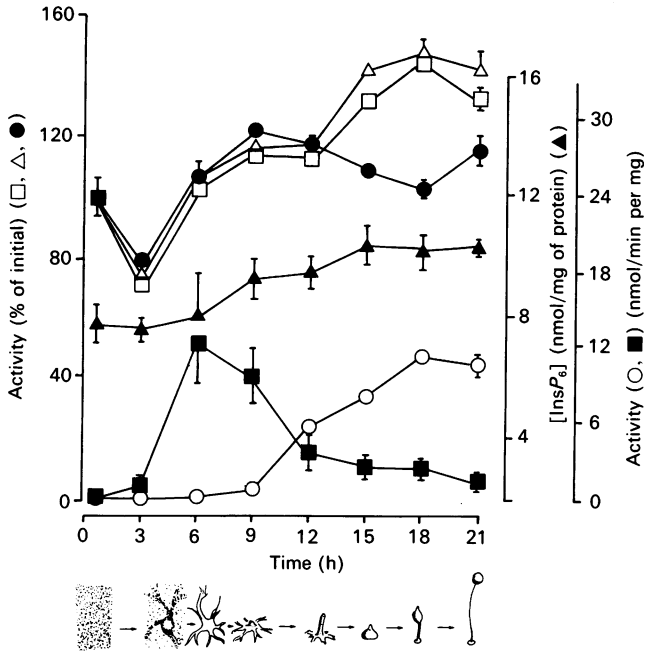

Fig. 13. Changes in the concentration of InsP_6 and in InsP_5 hydroxy kinase activities during development of *Dictyostelium*

An axenic strain of *Dictyostelium* (AX2) was starved of nutrients and plated out on solid agar supports to develop (see the Materials and methods section). Cells were harvested at various times to assay for InsP_6 concentration (▲), protein, cyclic AMP phosphodiesterase (■), glycogen phosphorylase (○) and [^{3}H]Ins(1,2,4,5,6)P_5 (△), [^{3}H]Ins(1,2,3,4,6)P_5 (□) and [^{3}H]Ins(1,3,4,5,6)P_5 (●) hydroxy kinase activities, as described in the Materials and methods section. A cartoon depiction of the approximate developmental stages of the organisms at the various times is given below the axis. InsP_6 concentrations were determined in a separate set of experiments (with their associated glycogen phosphorylase and cyclic AMP phosphodiesterase assays) and are presented as means ± S.E.M. ($n = 3$). The [^{3}H]InsP_5 kinase assays were performed on three independent sets of lysates each in duplicate; the data presented are based on means ± S.E.M. ($n = 6$) expressed as a proportion of the activity detected in amoebae ($t = 45$ min). The assays were performed under first-order conditions with respect to their [^{3}H]InsP_5 substrates exactly as described in the Materials and methods section (there was no significant dephosphorylation of internal [^{32}P]InsP_6 present in the assays). The activities measured in amoebae ($t = 45$ min) were: [^{3}H]Ins(1,2,3,4,6)P_5 hydroxy kinase, 2752 ± 93 d.p.m. into [^{3}H]InsP_6 in 30 min; D-[^{3}H]Ins(1,2,4,5,6)P_5 hydroxy kinase, 2020 ± 37 d.p.m.; [^{3}H]Ins(1,3,4,5,6)P_5 hydroxy kinase, 2966 ± 178 d.p.m. (all of the [^{3}H]InsP_5s possessed specific radioactivities of approx. 60 Ci/mmol). The marker enzymes cyclic AMP phosphodiesterase and glycogen phosphorylase were assayed in duplicate on two completely independent batches of amoebae (one of which yielded the InsP_6 data and one of which gave all the lysates which were assayed for [^{3}H]InsP_5 hydroxy kinase). The data were pooled and are presented as means ± S.E.M. ($n = 4$).

which had been introduced into the original acid extract. Although the marker enzymes and morphology of the slime-mould cultures went through the changes characteristic of normal differentiation and there was an appreciable alteration of the three [^{3}H]InsP_5 hydroxy kinase activities, the levels of InsP_6 only rose marginally (see Fig. 13).

DISCUSSION

Of the six possible InsP_5 isomers, at least five have been found in extracts prepared from a variety of cells. *Dictyostelium* extracts contained relatively similar concentrations of Ins(1,2,3,4,6)P_5, Ins(1,3,4,5,6)P_5 and D-Ins(1,2,4,5,6)P_5 (8–36 μM; Stephens & Irvine, 1990; see the Results section); HL60 and NG115-401L C3 cell extracts contained Ins(1,3,4,5,6)P_5 in a substantial excess (> 100-fold) over other InsP_5 species; and germinating mung-bean extracts contained readily detectable amounts of D- and L-Ins(1,2,4,5,6)P_5 (predominantly L-; see above) and D- and/or L-Ins(1,2,3,4,5)P_5. Clearly, a simple universal pattern of the relative abundance of InsP_5 isomers in cells is not emerging, although all these cell and tissue types contain substantial amounts of InsP_6. The reasons for the differences are unclear. There are huge phylogenetic differences between these different cell and tissue types, and also the germinating mung beans are unique among the group of cells studied in that they are in the process of rapidly dephosphorylating net amounts of InsP_6 (in at least one tissue compartment). One possible interpretation of the results is that there is a pattern which reflects the metabolism of InsP_6 (i.e. the accumulation of relatively large amounts of InsP_6 together with much lower levels of the InsP_5 isomers involved in InsP_6 biosynthesis and degradation), and within mammalian cells there is in addition the superimposition of an accumulation of one specific isomer of InsP_5, Ins(1,3,4,5,6)P_5. Such an interpretation would place emphasis on searching for possible distinct physiological functions for InsP_6 and Ins(1,3,4,5,6)P_5.

A survey of the InsP_5 hydroxy kinase activities in several different tissues also revealed a range of patterns. S. Biswas *et al.* (1978) have previously detected an enzyme with Ins(1,3,4,5,6)P_5 hydroxy kinase activity in highly purified fractions of mung beans (they would have considered it a consequence of an ADP: InsP_6 phosphotransferase operating in reverse). The data described above, obtained by an assay involving crude cytosolic fractions, suggest that, although other InsP_5 hydroxy kinase activities can also be detected in mung-bean supernatants, Ins(1,3,4,5,6)P_5 hydroxy kinase is the most active. The fact that [^{3}H]- or [^{32}P]-Ins(1,3,4,5,6)P_5 could not be detected in radiolabelled mung beans presumably means that its concentration is relatively low, and this would be consistent with the major InsP_5 isomer [L-Ins(1,2,4,5,6)P_5] detected in these preparations being a member of the pair of [^{3}H]InsP_5s that are least readily phosphorylated and therefore a prime candidate for membership of the pathway by which InsP_6 is dephosphorylated.

Dictyostelium amoebae contained InsP_5 hydroxy kinase activities capable of phosphorylating all three of their endogenous InsP_5s, D-Ins(1,2,4,5,6)P_5, Ins(1,3,4,5,6)P_5 and Ins(1,2,3,4,6)P_5, but not the three other InsP_5s (Stephens & Irvine, 1990; see above). However, only Ins(1,3,4,5,6)P_5 may act as a precursor in the synthesis of InsP_6 *de novo*, because, unlike both of the other InsP_5s, which are largely formed by dephosphorylation of InsP_6, it is the product of a series of five soluble *myo*-inositol and *myo*-inositol phosphate hydroxy kinase activities (Stephens & Irvine, 1990). Thus, of the two InsP_5 hydroxy kinase activities resolved in amoebae, one catalyses the phosphorylation of the InsP_5 involved in synthesis of InsP_6 *de novo* and the other catalyses the phosphorylation of the two InsP_5s involved in potential futile cycling of the phosphates at the 3- and 5-positions of InsP_6. The intracellular concentration of InsP_6 in amoebae (0.6 mM; Stephens & Irvine, 1990) is sufficient to cause substantial (80–90%) inhibition of all three InsP_5 hydroxy kinase activities, suggesting that these enzymes' potential to make InsP_6 *in vivo* is being substantially restrained by the levels of their product. This repressed capacity to manufacture InsP_6 could be readily expressed if the levels of InsP_6 were to fall and would therefore represent a buffering mechanism tending to hold InsP_6 levels constant. Such a stabilizing effect of this pattern of product inhibition might explain the lack of change in InsP_6 levels that occurs through the full developmental cycle of *Dictyostelium*. However, it should be emphasized that the assays on which the

effects of InsP_6 on the InsP_5 hydroxy kinase activities were seen were determined *in vitro*, and because the ionic form of InsP_6 in intact cells is unknown (and very probably both complex and critical to its properties) it is not clear that the InsP_5 hydroxy kinase activities would experience the same braking effect as that which would be expected from the assays *in vitro*. Nevertheless, given the profound changes that occur in cellular physiology and structure during the complete life cycle of *Dictyostelium*, it is a remarkable manifestation of the mechanisms responsible for InsP_6 homoeostasis in this organism that such an even level of InsP_6 is maintained throughout the entire process (Fig. 13).

In rat brain there are at least five InsP_5 hydroxy kinase activities, although we do not yet know how many distinct enzymes are involved. The initial rate of conversion of [^{3}H]Ins(1,3,4,5,6)P_5 into [^{3}H]InsP_6 is significantly smaller than for the other [^{3}H]InsP_5 hydroxy kinase activities. It is possible that the phosphorylation of [^{3}H]Ins(1,3,4,5,6)P_5 is severely limited by the presence of significant quantities of endogenous Ins(1,3,4,5,6)P_5 in the tissue. In this regard, the sole study of the InsP_5s that could be extracted from a mammalian brain only detected Ins(1,3,4,5,6)P_5, although this was with calf brains some time *post mortem*; the brains had been purchased from a local supermarket (Phillippy & Bland, 1988). We would predict, on the basis of the present results from mammalian cell lines, that Ins(1,3,4,5,6)P_5 would also be the major InsP_5 isomer in rat brain *in vivo*.

The number of different InsP_5 isomers and InsP_5 hydroxy kinase activities detected in cell extracts is somewhat bewildering. If these activities are an accurate representation of the routes of metabolism of InsP_5s and InsP_6 in intact cells, then these compounds are members of a metabolic network far more extensive and intricate than we had previously imagined and for which the physiological functions still remain obscure.

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