L-*myo*-Inositol 1,4,5,6-tetrakisphosphate (3-hydroxy)kinase

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Homogenates of primary-cultured murine bone macrophages contain an enzyme capable of synthesizing *myo*-[³H]inositol pentakisphosphate from *myo*-[³H]inositol tetrakisphosphate fractions derived from *myo*-[³H]inositol-labelled mouse macrophages and chick erythrocytes. D-*myo*-inositol 1,3,4,5-tetrakis[³²P]-phosphate present in the same incubations was not phosphorylated. Since the *myo*-[³H]inositol-labelled tetrakisphosphate fractions used as substrates consist of a mixture of L-*myo*-inositol 1,4,5,6-tetrakisphosphate (60–85 %) and a periodate-resistant tetrakisphosphate(s) whose characteristics are consistent with those of D-*myo*-inositol 1,3,4,5-tetrakisphosphate [the preceding paper [Stephens, Hawkins, Carter, Chahwala, Morris, Whetton & Downes (1988) *Biochem. J.* 249, 271–282]], these data suggest the existence of a kinase that phosphorylates L-*myo*-inositol 1,4,5,6-tetrakisphosphate to give a *myo*-inositol pentakisphosphate. A similar activity was identified in homogenates of rat cerebrum, liver, heart and parotid gland. D-*myo*-inositol 1,3,4,5-tetrakis[³²P]phosphate in the same incubations was not a substrate. The activity was almost entirely soluble in all the tissues investigated and was found at its greatest specific activity in brain cytosol. The activity was purified 120-fold from a rat brain homogenate by (NH₄)₂SO₄ fractionation and anion-exchange chromatography. The activity was clearly distinct from D-*myo*-inositol 1,4,5-trisphosphate (3-hydroxy)kinase. Incubation of this partially purified preparation with L-*myo*-[³H]inositol 1,4,5,6-tetrakisphosphate from chick erythrocytes and [γ-³²P]ATP resulted in the formation of L-*myo*-[³H]-inositol [1-³²P]1,3,4,5,6-pentakisphosphate. The enzyme is therefore identified as an L-*myo*-inositol 1,4,5,6-tetrakisphosphate (3-hydroxy)kinase.

INTRODUCTION

myo-Inositol pentakisphosphate (InsP₅) has been identified in a number of vertebrate cells (for a review, see Bartlett, 1980). The structure of this compound in chicken erythrocytes is Ins(1,3,4,5,6)P₅ (Johnson & Tate, 1969). This assignment was achieved through the application of ³¹P-n.m.r. techniques. Although ³¹P-n.m.r. can be totally definitive when handling molecules with a plane of symmetry [e.g. Ins(1,3,4,5,6)P₅] or in the presence of a specific chiral shifting reagent, this type of analysis requires several micromoles of homogeneous material. This limitation represents a barrier that has severely hampered the study of InsP₅ species in tissues or cells, where their concentrations are substantially below that in avian erythrocytes. Moreover, because they are resistant to oxidation by periodate, the structure of trace quantities of radiolabelled InsP₅ species cannot be obtained by the traditional method of periodate oxidation followed by reduction, dephosphorylation and chromatographic identification of the resulting polyol (Grado & Ballou, 1961).

The metabolism of InsP₅ is similarly poorly defined. Isaacs & Harkness (1976) have shown that, if avian erythrocytes are incubated *in vitro* with [¹⁴C]Ins, the tracer is eventually incorporated into InsP₅. This is presumably a consequence of ongoing metabolic turnover. The route by which InsP₅ is synthesized and broken down in this presumed turnover cycle is unknown.

Two InsP₄ species have been identified in cells, namely D-Ins(1,3,4,5)P₄ (Batty *et al.*, 1985) and L-Ins(1,4,5,6)P₄ (Stephens *et al.*, 1988). The experiments described here were designed to investigate the metabolic relationship between these InsP₄ species and InsP₅. They demonstrate that L-Ins(1,4,5,6)P₄, but not D-Ins(1,3,4,5)P₄, is a substrate for a widely distributed cytosolic inositol phosphate kinase responsible for the synthesis of Ins(1,3,4,5,6)P₅.

MATERIALS AND METHODS

Preparation of homogenates

Primary cultures of mouse bone macrophages were prepared as described in the preceding paper (Stephens *et al.*, 1988). The cells were cultured on 10-cm-diameter dishes and harvested for homogenization by rapidly aspirating the medium, then washing the adherent macrophages with 50 ml of 10 mM-Hepes/2 mM-EGTA, pH 7.0 at 4 °C, containing PMSF (final concn. 50 μM), antipain, leupeptin and pepstatin (all 1 μg/ml final concn.). This buffer was immediately removed and the cells allowed to lyse, on ice, in the remnants of the medium. After 5 min the cells were completely lysed. They were then harvested with a silicone wedge and finally disrupted with five strokes in a tight-fitting Teflon-on-glass hand homogenizer. This homogenate

Abbreviations used: Ins, *myo*-inositol; L-Ins(1,4,5,6)P₄, L-*myo*-inositol 1,4,5,6-tetrakisphosphate; D-Ins(1,3,4,5)P₄, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; D-Ins(1,4,5)P₃, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1,3,4,5,6)P₅, *myo*-inositol 1,3,4,5,6-pentakisphosphate; PtdIns, D-phosphatidyl-*myo*-inositol; PtdIns4P, D-phosphatidyl-*myo*-inositol 4-phosphate; PtdIns(4,5)P₂, D-phosphatidyl-*myo*-inositol 4,5-bisphosphate; PAF, platelet-activating factor; DTT, dithiothreitol; POPOP, 1,4-bis-(5-phenyloxazol-2-yl)benzene; PPO, 2,5-diphenyloxazole; PMSF, phenylmethanesulphonyl fluoride; BSA, bovine serum albumin; f.p.l.c., fast protein liquid chromatography.

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was used immediately and typically possessed a protein concentration of 4–5 mg/ml.

Homogenates of rat cerebrum, liver, parotid gland and heart were prepared using one to ten 200 g male rats, depending on the quantity of enzyme required. The tissues were removed rapidly from stunned rats and rinsed with ice-cold homogenization buffer (see below), weighed, and then disrupted with ten strokes on a motor-driven Potter–Elvehjem homogenizer at a tissue density of approx. 1 g/5 ml of buffer. The homogenization buffer contained 0.25 M-sucrose, 50 mM-Hepes, 2 mM-EGTA, pH 7.0 at 4 °C, PMSF (50 μ M final concn. from a 50 mM stock in propan-2-ol), leupeptin, pepstatin and antipain (all 1 μ g/ml final concn. from 1 mg/ml stocks in methanol). The crude homogenates were filtered through a tea-strainer and then used immediately.

Preparation of soluble and particulate fractions from tissue homogenates

Aliquots (1 ml) of macrophage homogenate were centrifuged at 100 000 rev./min at 4 °C for 10 min (Beckman TL-100 ultracentrifuge). The soluble fraction produced was used directly; the pellet was resuspended to the volume of the initial homogenate with lysis buffer.

Small volumes of homogenates from rat tissues were handled as described above for macrophages. In preparative experiments, the homogenate from ten rat brains was centrifuged at 50 000 rev./min for 25 min in 20 ml aliquots (at 4 °C in a Beckman L δ -M centrifuge). The soluble fraction was used directly, and a particulate fraction was prepared as described above.

H.p.l.c.

Three varieties of anion-exchange column were utilized (all purchased from Jones Chromatography, Hengoed, Mid-Glamorgan, Wales, U.K.). A Partisil 10-SAX column (25 cm $\times$ 0.46 cm main column; 2 cm $\times$ 0.46 cm guard column) was used to separate the more highly phosphorylated inositol phosphates. This column was eluted with a gradient based on water and 3.5 M-ammonium formate (adjusted to pH 3.7 at 22 °C with H₃PO₄) as described in the preceding paper (Stephens *et al.*, 1988).

A Partisil 5-PAC column (25 cm $\times$ 0.46 cm main column; 2 cm $\times$ 0.46 cm guard column) was used to separate inositol monophosphates. This column was pre-equilibrated with propan-2-ol (100 ml at 0.5 ml $\cdot$ min⁻¹), then with water (100 ml at 0.75 ml $\cdot$ min⁻¹) and finally with the chromatography solvent (see below). The column was eluted with a gradient based on (A) water and (B) 1.2 M-sodium acetate/acetic acid, pH 3.6 at 22 °C, as follows: 0 min, 0% B; 5 min, 0% B; 45 min, 50% B; flow rate 0.75 ml $\cdot$ min⁻¹ (the system had a void time, from the mixing-chamber inlets to the fraction collector, of 12.75 min under these conditions). This column was characterized by application of a mixture of Ins2P (Sigma), L-[¹⁴C]Ins1P (Amersham), D/L-[³H]-Ins1P and [³H]Ins2P (prepared as described by Siess, 1985) and [³²P]P_i. Baseline separation of D/L-Ins1P and Ins2P was achieved using the gradient described above.

A Vydac nucleotide column was used to purify [γ -³²P]ATP. This column was eluted at 1.25 ml $\cdot$ min⁻¹ with a gradient based on (A) water and (B) 0.7 M-

potassium phosphate/88 mM-H₃PO₄ (pH 2.85 at 22 °C; 0 min, 0% B; 5 min, 0% B; 40 min, 100% B).

H.p.l.c. fractions were desalted as described previously (Downes *et al.*, 1986).

Preparation of substrates

L-*myo*-[³H]Ins(1,4,5,6)P₄ was prepared as described in the preceding paper (Stephens *et al.*, 1988). The strategy employed was to label chick erythrocytes with *myo*-[³H]-inositol for 1–2 days and extract, purify (by h.p.l.c.) and desalt a [³H]InsP₄ fraction. This material was used as a routine assay substrate. It contained 70% of the total radioactivity as L-Ins(1,4,5,6)P₄ [as measured by periodate oxidation, followed by reduction, dephosphorylation and identification and quantification of the resulting polyols; see the preceding paper (Stephens *et al.*, 1988)]. The remaining 30% of the radioactivity was found in a periodate-resistant [³H]InsP₄ that did not act as a substrate for the InsP₄ kinase (see below). Data throughout the present paper have been corrected, where appropriate, for the quantity of periodate-resistant [³H]-InsP₄ in the substrate preparation, i.e. when the values for the percentage of substrate converted are quoted, this refers to the proportion of the periodate-sensitive [³H]-InsP₄ that had been phosphorylated.

[³H]InsP₄ from [³H]Ins-prelabelled murine macrophages was prepared as described in the preceding paper (Stephens *et al.*, 1988).

[γ -³²P]ATP was purchased from Amersham International [triethylammonium salt; 'stabilized' solution]. Before this could be used as a substrate (see below), however, it had to be purified to remove large quantities of contaminating [³²P]polyphosphate compounds. This purification was achieved by applying 10 nmol of carrier ATP plus [γ -³²P]ATP to a Vydac nucleotide column, followed by elution with a linear gradient of potassium phosphate/H₃PO₄ (see above). The absorbance of the eluate at 259 nm was continuously monitored, and 20-drop fractions were collected. ATP was eluted after 18 min in approx. 0.28 M-potassium phosphate/35.2 mM-H₃PO₄. The peak [γ -³²P]ATP fraction was neutralized (with 37 μ l of 2 M-KOH/0.2 M-Hepes) and used immediately. Potassium phosphate at 30 mM did not significantly alter the activity of InsP₄ kinase.

D-Ins[³²P](1,4,5)P₃ and D-Ins[³²P](1,3,4,5)P₄ were prepared as described in the preceding paper (Stephens *et al.*, 1988).

Ins[³²P]P₅ and L-Ins[³²P](1,4,5,6)P₄ were prepared from [³²P]P_i-labelled chick erythrocytes. The erythrocytes were prepared as described in the preceding paper (Stephens *et al.*, 1988) and incubated with 2 mCi of [³²P]P_i (Amersham International; carrier-free). After 32 h of incubation the cells were lysed, precipitated with HClO₄, and an aqueous extract prepared by sedimenting the cell debris by centrifugation then neutralizing the supernatant with tri-*n*-octylamine/Freon (Sharpes & McCarl, 1982). The aqueous extract was treated with 50 mg of Norit-GSX charcoal in 10% (w/v) trichloroacetic acid. After mixing, the extract was left on ice for 15 min. A 2 mg portion of BSA was added to the mixture, and the charcoal was pelleted by centrifugation. The trichloroacetic acid was removed from the supernatant with 1.1 vol. of tri-*n*-octylamine/Freon (1:1, v/v). Ins[³²P]P₅ was purified from the nucleotide-free aqueous extract by h.p.l.c. with a Partisil 10-SAX h.p.l.c. column

as described in the preceding paper (Stephens *et al.*, 1988). The fractions containing the $\text{Ins}^{[32]\text{P}}\text{P}_5$ were pooled, neutralized with triethylamine and desalted as described by Downes *et al.* (1986).

^{14}C - and ^3H -labelled $\text{Ins}2\text{P}$ and $\text{D/L-Ins}1\text{P}$ were prepared as follows. $\text{Ptd}^{[3\text{H}]\text{Ins}}$ ($1\ \mu\text{Ci}$; Amersham International) or $\text{Ptd}^{[14\text{C}]\text{Ins}}$ ($0.2\ \mu\text{Ci}$; Amersham International) were dried under N_2 . A $200\ \mu\text{l}$ portion of $0.1\ \text{M-HCl}$ was added to the tube and the mixture was boiled for 40 min (Pizer & Ballou, 1959; Siess, 1985). The resulting solution was extracted with light petroleum (b.p. $40\text{--}60\ ^\circ\text{C}$), neutralized with $2\ \text{M-KOH}/0.2\ \text{M-Hepes}$ and diluted 10-fold with water. A $10\ \mu\text{l}$ portion of $0.1\ \text{M-EDTA}$, pH 7.0, was then added, and this sample was used directly as a chromatographic standard. This process, of boiling PtdIns in $0.1\ \text{M-HCl}$, yields approximately equal amounts of $\text{D-Ins}1\text{P}$, $\text{L-Ins}1\text{P}$ and $\text{Ins}2\text{P}$ (Pizer & Ballou, 1959). $\text{D-}^{[3\text{H}]\text{Ins}}(1,4,5)\text{P}_3$ was purchased from Amersham.

Protein-separation techniques

The supernatant produced by centrifugation of rat brain homogenates was differentially precipitated with $(\text{NH}_4)_2\text{SO}_4$. The precipitations were carried out on ice and percentage-saturation values quoted are for solutions at $0\ ^\circ\text{C}$. Upon addition of the $(\text{NH}_4)_2\text{SO}_4$ the fractions were left for 20 min on ice, after which time they were centrifuged at $15000\ \text{rev./min}$ ($39000\ g$) for 20 min in a refrigerated centrifuge (Beckman JS-21). The protein precipitates were solubilized by diluting them approx. 10-fold into $50\ \text{mM-Hepes}/2\ \text{mM-EGTA}/15\ \text{mM-2-mercaptoethanol}/2\ \text{mM-DTT}$, pH 7.0 at $4\ ^\circ\text{C}$, and were then dialysed against 1000 vol. of the same buffer for at least 3 h.

Proteins in the $40\text{--}50\%$ -satd. $(\text{NH}_4)_2\text{SO}_4$ fraction (see above) were separated by anion-exchange chromatography on a Mono-Q H/R 10/10 analytical f.p.l.c. column. The column was pre-equilibrated with $50\ \text{mM-Tris}/\text{HCl}/1\ \text{mM-EGTA}$, pH 7.2 at $4\ ^\circ\text{C}$, for 1 h. Approx. $5\text{--}10\ \text{mg}$ of protein was applied to the column in a volume of 1 ml (see the Results section). The protein was eluted with the following gradient: 0 min, 0% B (B = $1\ \text{M-NaCl}/50\ \text{mM-Tris}/\text{HCl}/1\ \text{mM-EGTA}$, pH 7.2 at $4\ ^\circ\text{C}$); 5 min, 0% B; 50 min, 50% B; stepping to 50 min, 100% B; 60 min, 100% B; the flow rate was $0.5\ \text{ml}\cdot\text{min}^{-1}$ and the absorbance of the eluate at 280 nm was monitored continuously. Fractions were collected every 1 min, and $10\ \mu\text{l}$ of each fraction was assayed for $\text{L-}^{[3\text{H}]\text{Ins}}(1,4,5,6)\text{P}_4$ kinase and phosphatase (see below) and $\text{D-}^{[3\text{H}]\text{Ins}}(1,4,5)\text{P}_3$ kinase (see below). The fractions containing InsP_4 kinase activity were pooled and stored as described in Fig. 2 (below).

The M_r of the native enzyme was estimated by gel filtration through a calibrated Superose-12 gel-filtration f.p.l.c. column (Pharmacia). A $200\ \mu\text{l}$ portion of the redissolved $40\text{--}50\%$ -satd. $(\text{NH}_4)_2\text{SO}_4$ fraction from rat brain cytosol (in $50\ \text{mM-Hepes}/2\ \text{mM-EGTA}/2\ \text{mM-DTT}/15\ \text{mM-2-mercaptoethanol}$, pH 7.0 at $4\ ^\circ\text{C}$) was dialysed for 12 h against $0.5\ \text{M-KCl}/50\ \text{mM-Hepes}/2\ \text{mM-EGTA}/2\ \text{mM-DTT}/15\ \text{mM-2-mercaptoethanol}$, then applied to a Superose-12 f.p.l.c. column that had been pre-equilibrated with the dialysis buffer for 2 h. The flow rate was $0.3\ \text{ml}\cdot\text{min}^{-1}$. The absorbance of the eluate was monitored continuously at 280 nm, and fractions were collected from V_0 every 1.67 min for 1 h ($V_0 = 7.75\ \text{ml}$; $V_t = 18.8\ \text{ml}$). Portions ($10\ \mu\text{l}$) of each fraction were

assayed for $\text{L-Ins}}(1,4,5,6)\text{P}_4$ kinase activity as described below. M_r standards were run under similar conditions.

Assay of $\text{D-myoin}}(1,4,5)\text{P}_3$ kinase

$\text{D-Ins}}(1,4,5)\text{P}_3$ kinase was assayed under first-order-kinetic conditions. A $10\ \mu\text{l}$ sample of each fraction was added to an assay tube containing 50000 d.p.m. of $\text{D-}^{[3\text{H}]\text{Ins}}(1,4,5)\text{P}_3$ (Amersham International), $6\ \text{mM-MgCl}_2$, $5\ \text{mM-ATP}$, $50\ \text{mM-Hepes}$, $2\ \text{mM-EGTA}$, $50\ \text{mM-KCl}$ and $5\ \text{mM-2-mercaptoethanol}$, pH 7.2 at $37\ ^\circ\text{C}$, in a final volume of $200\ \mu\text{l}$. After 15 min the assays were terminated with $0.5\ \text{ml}$ of ice-cold 5% (w/v) HClO_4 , which was subsequently extracted with 1.1 vol. of tri-*n*-octylamine/Freon (1:1, v/v). The $^{[3\text{H}]}$ inositol phosphate species were separated by batch elution from anion-exchange-resin columns (see below).

Assay of $\text{L-myoin}}(1,4,5,6)\text{P}_4$ kinase

Assays were in a final volume of $200\ \mu\text{l}$ containing $5\ \text{mM-ATP}$, $10\ \text{mM-phosphocreatine}$, $0.494\ \text{mM-CaCl}_2$, $5.91\ \text{mM-MgSO}_4$, $2\ \text{mM-EGTA}$, $50\ \text{mM-Hepes}$, $100\ \text{mM-KCl}$, $4\ \text{mM-DTT}$, pH 7.0 at $37\ ^\circ\text{C}$. This gave a measured free ionized Ca^{2+} concentration of $250\ \text{nM}$ (estimated with the Ca^{2+} -sensitive dye Fura-II) and a theoretical free Mg^{2+} concentration of $1\ \text{mM}$. In work with homogenates, particulate and soluble fractions, creatine kinase (Sigma) $5\ \text{units/ml}$ was included in the assay mixture to maintain ATP levels (this preparation contained no detectable $^{[3\text{H}]\text{Ins}}(1,4,5,6)\text{P}_4$ kinase activity). Partially purified fractions were assayed without phosphocreatine kinase. $\text{L-}^{[3\text{H}]\text{Ins}}(1,4,5,6)\text{P}_4$ was usually added at a concentration of 4000 d.p.m./ $200\ \mu\text{l}$ of assay mixture, representing a concentration of roughly $0.5\ \mu\text{M}$. Portions ($10\text{--}40\ \mu\text{l}$) of homogenate, soluble or particulate fractions (or subfractions of these) were added to this assay mixture. Incubations were normally for 10 min at $37\ ^\circ\text{C}$, and under these circumstances less than 50% of the available substrate was consumed, and the assay operated under first-order-kinetic conditions with respect to the quantity of enzyme activity present. ATPase activity was estimated by 'spiking' the reaction mixture with a tracer quantity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (without phosphocreatine kinase). The production of $^{[32}\text{P}]\text{P}_i$ was measured after batch elution from anion-exchange resin (see below). In some experiments $\text{Ins}^{[32}\text{P}]\text{P}_5$ or $\text{D-Ins}^{[32}\text{P}](1,3,4,5)\text{P}_4$ was also added to the assay mixtures.

In experiments in which the total ATP concentration was decreased to $0.3\ \text{mM}$ (e.g. when preparing $\text{Ins}^{[32}\text{P}]\text{P}_5$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; this was the lowest concentration of ATP found to maintain full activity of InsP_4 kinase; results not shown), the quantity of MgSO_4 was raised to $6.0\ \text{mM}$ and that of CaCl_2 reduced to $0.2\ \text{mM}$. This was done to alleviate the rise in free Ca^{2+} caused by the reduction in ATP, which inhibits the kinase by up to 40% under these conditions (results not shown).

Reactions were terminated by the addition of $400\ \mu\text{l}$ of ice-cold 4% (w/v) HClO_4 , containing phytic acid ($250\ \mu\text{g/ml}$) to act as a carrier. Precipitated protein was pelleted by centrifugation in a bench-top centrifuge (maximum setting for 5 min). The supernatant was removed and mixed with 1.1 vol. of tri-*n*-octylamine/Freon (1:1, v/v), then centrifuged to separate the three phases present. A $50\ \mu\text{l}$ portion of $0.1\ \text{M-EDTA}$, pH 7.0, was added to the upper phase before diluting it 10-fold with water and applying this to a $2\ \text{cm}\times 0.6\ \text{cm}$ column of Bio-Rad AG-1 anion-exchange resin (X8; $200\text{--}400$

mesh; formate form). Fractions eluted (and solvents used) were: free Ins, 10 ml of water; InsP (and [32 P]P_i), 9 ml of 0.2 M-ammonium formate/0.1 M-formic acid; InsP₂, 9 ml of 0.4 M-ammonium formate/0.1 M-formic acid; InsP₃, 10 ml of 0.8 M-ammonium formate/0.1 M-formic acid; InsP₄ (and [32 P]ATP), 10 ml of 1.05 M-ammonium formate/0.1 M-formic acid; InsP₅, 6 ml of 2.0 M-ammonium formate/0.1 M-formic acid. The resulting fractions were dissolved in a scintillation-fluid cocktail (1 vol. of Triton-X-100/xylene (1:1, v/v), containing 15 g of PPO and 0.375 g of POPOP/2 litres of xylene) before counting for radioactivity.

Recoveries of the two previously uncharacterized inositol phosphates through this process were: Ins[32 P]P₅, 92 % (mean of 20 independent values); [3 H]InsP₄, 93 % (mean of 45 independent values). In all, 96 % of recovered [3 H]InsP₄ and Ins[32 P]P₅ was eluted in the appropriate fractions designated above.

Preparation of [3 H]Ins[32 P]P₅

A total of 180 000 d.p.m. of L-[3 H]Ins(1,4,5,6)P₄ was incubated with rat brain InsP₄ kinase in the presence of 20 mM-KCl, 5 mM-DTT, 5 mM-2-mercaptoethanol, 2 mM-EGTA, 1.3 mM-MgSO₄, 0.2 mM-CaCl₂, 0.3 mM-[γ - 32 P]ATP (1.6 μ Ci/nmol), 30 mM-potassium phosphate (this was derived from the h.p.l.c. eluate in which the [γ - 32 P]ATP was added), 50 mM-Hepes, pH 7.0 at 37 °C, in a final volume of 4 ml. The InsP₄ kinase was added in 200 μ l of 50 % (v/v) glycerol from a preparation obtained after chromatography on a Mono-Q column (see above and the legend to Fig. 2 below); this represented the total activity found in the cytosol fractions from one or two rat brains; it contained 140 μ g of protein and was capable of phosphorylating 100 000 d.p.m. of the [3 H]-InsP₄ substrate in 10 min. After 60 min incubation at 37 °C, 200 μ l of hexokinase solution was added. This was prepared by using 100 μ l of hexokinase in an (NH₄)₂SO₄ suspension (Boehringer Mannheim), which was pelleted by centrifugation, the supernatant removed, and the sediment dissolved in 200 μ l of 0.4 M-glucose/20 mM-Hepes, pH 7.0. The incubation was continued for a further 12 min at 37 °C, after which time the [32 P]ATP had been quantitatively converted into glucose 6-[32 P]phosphate (results not shown; [3 H]InsP₅ was not dephosphorylated or phosphorylated during this process). This step was included to decrease the background 32 P that was eluted from a Partisil 10-SAX h.p.l.c. column with a retention time similar to InsP₄ and InsP₅. The reaction was terminated with 120 μ l of 0.1 M-EDTA, pH 7.0, and the mixture immediately filtered and applied to a Partisil 10-SAX h.p.l.c. column (a 10 ml loading loop was constructed to enable this to be accomplished in one step), and the column was eluted as described above. The absorbance of the column eluate was monitored continuously at 259 nm. The eluate was collected in 0.4 min fractions, which were counted for Čerenkov radiation in a liquid-scintillation counter. Two major 32 P-labelled peaks were eluted later than 40 min. One was eluted at the time expected for InsP₅ (54–55 min) and contained 3 H radioactivity. The other peak of 32 P was eluted with a slightly shorter retention time, did not contain 3 H radioactivity and was present in zero-time incubation mixtures. This latter compound is probably a [32 P]polyphosphate. The peak [3 H]Ins[32 P]P₅ fractions were pooled, neutralized with triethylamine

and desalted as described above. The desalted [3 H]Ins[32 P]P₅ fraction was then re-chromatographed on a Partisil 10-SAX h.p.l.c. column. The [3 H]Ins[32 P]P₅ fraction was again desalted as described above, with one exception: at the final chromatography step a Bio-Rad AG-50W (NH₄⁺ form) cation-exchange column was employed, thus yielding the ammonium salt of [3 H]-Ins[32 P]P₅. The final yield of [3 H]Ins[32 P]P₅ was 0.75 μ Ci of 32 P radioactivity (this contained 0.01 μ Ci of 3 H radioactivity).

To assess the final purity of the [3 H]Ins[32 P]P₅ preparation, a portion was mixed with a relatively large quantity of [3 H]Ins(1,3,4,5,6)P₅ and applied to a Partisil 10-SAX h.p.l.c. column, which was eluted as described above. In all, 82 % of the 32 P radioactivity in the preparation co-chromatographed precisely with the 3 H-labelled standard. There were two 32 P-labelled contaminants, amounting to 18 % of the total radioactivity, which migrated in the positions expected of [32 P]P_i and [32 P]pyrophosphate. When subjected to NH₃ hydrolysis (see below) these contaminants yielded only [32 P]P_i (results not shown).

For the partial hydrolysis of [3 H]Ins[32 P]P₅ with NH₃ (see below), a mixture containing the above preparation of the ammonium salt of [3 H]Ins[32 P]P₅ and that of Ins(1,3,4,5,6)P₅ (to act as carrier for the smaller quantity of radioactive material) was made. This mixture was prepared as follows: 1 mg of the barium salt of Ins(1,3,4,5,6)P₅ (Calbiochem) was dissolved by adding a slurry containing 100 μ l of Bio-Rad AG-50W resin (H⁺ form). The resulting solution was applied to a column containing 2 ml of Bio-Rad AG-50W resin (NH₄⁺ form). The column was then washed with water, and the pooled eluate freeze-dried. The dried residue was redissolved in a small volume of water and added to the [3 H]Ins[32 P]P₅ preparation. A total of 140 000 32 P d.p.m. of this mixture was added to a mixture of [14 C]inositol phosphate standards, applied to a Partisil h.p.l.c. column (see above) and eluted as described above. This sample acted as a control for a further sample of the mixture, which underwent NH₃ hydrolysis (see below).

Partial hydrolysis of [3 H]Ins[32 P]P₅

A total of 201 000 32 P d.p.m. of the ammonium salt of [3 H]Ins[32 P]P₅ [containing about 0.5 mg of the ammonium salt of Ins(1,3,4,5,6)P₅; see above] was dried under vacuum, dissolved in 0.4 ml of aq. 10 M-NH₃ sealed in a Carius tube and warmed to 110 °C in a thermostatically controlled heating block (Tomlinson & Ballou, 1961). After 100 h (this time was found to be optimal for the production of inositol monophosphates from InsP₅; results not shown), the Carius tube was broken open and the contents freeze-dried. The material remaining was dissolved in 1 ml of 20 mM-EDTA, pH 7.0, and a portion mixed with D/L-[14 C]Ins1P and [14 C]Ins2P (prepared as described above) and applied to a Partisil 5-PAC h.p.l.c. column.

Organic-phosphate assay

Organic phosphate was assayed by the method of Bartlett (1959), with one modification. The anilino-naphthalenesulphonate reagent was replaced by a solution of 10 % (w/v) ascorbate (this modification requires the assay tubes to be rapidly cooled on ice after the final colour-developing reaction has reached completion).

Fig. 1. Phosphorylation of InsP_4 species by macrophage homogenates

A macrophage homogenate (4.5 mg of protein/ml) was prepared as described in the Materials and methods section. Aliquots (200 μl) of this homogenate were incubated in buffer containing an ATP-regenerating system (see the Materials and methods section) and (a) [^3H] InsP_4 prepared from chicken erythrocytes (6625 d.p.m. added, of which 102 % was recovered) and D- Ins [^{32}P](1,3,4,5) P_4 (586 c.p.m. added, of which 97 % was recovered) for 30 min, or (b) [^3H] InsP_4 prepared from unstimulated macrophages (1000 d.p.m. added, of which 88 % was recovered) and D- Ins [^{32}P](1,3,4,5) P_4 (523 c.p.m. added, of which 93 % was recovered) for 18 min, or (c) [^3H] InsP_4 prepared from PAF (2 $\mu\text{g}/\text{ml}$ final concn.)-stimulated macrophages (9630 d.p.m. added, of which 100 % was recovered) and D- Ins [^{32}P](1,3,4,5) P_4 (812 c.p.m. added, of which 94 % was recovered) for 18 min. Incubations were terminated and neutralized extracts were separated by h.p.l.c. on Partisil 10-SAX as described in the Materials and methods section. Zero-time incubations contained single peaks of ^3H and ^{32}P radioactivity (results not shown).

Table 1. L-Ins^[32P](1,4,5,6)P₄ metabolism in macrophage homogenates

Each assay mixture contained a mean of 1780 d.p.m. of L-Ins^[32P](1,4,5,6)P₄ (from chick erythrocytes; see the Materials and methods section) and 240 µl of the cell fraction indicated (the homogenate contained 4.0 mg of protein/ml). The assay conditions were as described in the Materials and methods section. Acid extracts of each of the incubations were neutralized and separated on a Partisil 10-SAX h.p.l.c. column.

	Incubation time (min)	³² P radioactivity in fraction (c.p.m.)					Proportion recovered
		[³² P]P _i	Ins[³² P]P ₃	Ins[³² P]P ₄	Ins[³² P]P ₅	Total	
Macrophage homogenate, with ATP	40	170	90	1150	310	1720	0.88
Soluble fraction, with ATP	40	90	70	1380	295	1835	0.90
Particulate fraction, with ATP	40	215	105	1445	25	1790	0.86
Soluble, dialysed, with ATP	40	95	65	1400	280	1840	0.92
Soluble, dialysed, with ATP	20	55	50	1510	150	1765	0.90
Soluble, dialysed, no ATP	40	100	65	1580	5	1750	0.87
Soluble, dialysed, 90 °C for 5 min, with ATP	20	20	10	1715	5	1750	0.87

Table 2. Tissue distribution of InsP₄ kinase activity in the rat

A mean of 1550 d.p.m. of [³H]InsP₄ (from chick erythrocytes; see the Materials and methods section) was included in every assay. The maximum proportion of the available substrate that was phosphorylated was 50 % (brain soluble fraction). The maximum proportion of total substrate dephosphorylated was 7 % (liver homogenate). Ins[³²P]P₅ was included in every assay, and the maximum proportion dephosphorylated was 8 %. The assay was run for 10 min at 37 °C (for further details, see the Materials and methods section). All data are means of duplicates.

Tissue	InsP ₄ kinase activity (d.p.m. in InsP ₅ produced · 10 min ⁻¹ · mg of protein ⁻¹)	Protein (µg/assay)
Brain		
Homogenate	858	512
Soluble	4316	120
Particulate	144	452
Parotid		
Homogenate	259	224
Soluble	485	140
Particulate	20	96
Liver		
Homogenate	458	900
Soluble	995	400
Particulate	29	620
Heart		
Homogenate	160	284
Soluble	500	156
Particulate	0	156

Protein assay

The method of Bradford (1976) was used, with BSA as a standard.

RESULTS AND DISCUSSION

Addition of InsP₄ species to primary-cultured mouse bone macrophage homogenates

In the preceding paper (Stephens *et al.*, 1988) we showed that the predominant species of InsP₄ present in

both avian erythrocytes and unstimulated macrophages is L-Ins(1,4,5,6)P₄. Samples of [³H]InsP₄ purified from either of these sources (containing 70 % L-Ins(1,4,5,6)P₄ in the case of the avian erythrocyte and 40–85 % L-Ins(1,4,5,6)P₄ in the case of the macrophage InsP₄ fraction; see Stephens *et al.*, 1988) were mixed with D-Ins[³²P](1,3,4,5)P₄ and incubated with a macrophage homogenate, in a buffer containing an ATP-regenerating system, for various times. Acid extracts of the resulting reaction mixtures were then separated by h.p.l.c. (see Fig. 1).

The D-Ins[³²P](1,3,4,5)P₄ was hydrolysed by the homogenate to yield compounds with the chromatographic properties expected of D-Ins[³²P](1,3,4)P₃, Ins[³²P]P₂ and [³²P]P_i (Batty *et al.*, 1985; Hansen *et al.*, 1986; Hawkins *et al.*, 1986; Shears *et al.*, 1987). A much lower proportion of the [³H]InsP₄ fraction derived from chick erythrocytes (see Fig. 1a) or unstimulated macrophages (see Fig. 1b) was hydrolysed in the same incubations, although some less polar metabolites were clearly produced. However, a [³H]Ins-labelled compound with the precise chromatographic properties expected of InsP₅ was produced. There was no ³²P radioactivity associated with this [³H]Ins-labelled compound, and it accumulated linearly with time up to 40 min of incubation (see Table 1).

The result of the same type of experiment using a [³H]InsP₄ fraction derived from hormone-stimulated macrophages is shown in Fig. 1(c). This sample contained 6 % L-Ins(1,4,5,6)P₄, with the remaining InsP₄ being periodate-resistant. PAF stimulation for 30 s results in a 20-fold increase in the periodate-resistant InsP₄, with no detectable effect upon the L-Ins(1,4,5,6)P₄ (Stephens *et al.*, 1988). The results shown in Fig. 1(c) were obtained by using an InsP₄ fraction obtained from the same quantity of macrophages as that used to obtain the results in Fig. 1(b). Hence it contained an equivalent amount of L-[³H]Ins(1,4,5,6)P₄, but approx. 20 times more of the periodate-resistant InsP₄. A much greater proportion of this sample was dephosphorylated (Fig. 1c cf. Fig. 1b), but approximately the same amount of [³H]InsP₅ was formed. These results suggest that an enzyme in macrophage homogenates can phosphorylate L-Ins(1,4,5,6)P₄, but not D-Ins(1,3,4,5)P₄, or any other periodate-insensitive InsP₄ that may be present in macrophages stimulated with PAF for 30 s, to yield an InsP₅ species.

Fig. 2. Separation of rat brain cytosol InsP_4 kinase on a Mono-Q anion-exchange f.p.l.c. column

Protein (5.5 mg) from a 40–50 % -satd. $(\text{NH}_4)_2\text{SO}_4$ fraction of rat brain cytosol (see the Materials and method section) was applied to a Mono-Q H/R 10/10 analytical anion-exchange f.p.l.c. column and eluted with a gradient of solution B (.....) as shown in the Figure. The A_{280} of the eluate was monitored continuously (—; 0 to a full scale of 1.0) and 0.5 ml fractions were collected for 60 min. A sample of each fraction was assayed for D- $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ kinase (Δ), $[\text{^3H}]\text{InsP}_4$ kinase ($\blacktriangle$) and $[\text{^3H}]\text{InsP}_4$ phosphatase ($\square$) activities (see the Materials and methods section). In all, 70 % of the D- $\text{Ins}(1,4,5)\text{P}_3$ kinase, 92 % of the InsP_4 kinase and 90 % of the InsP_4 phosphatase activities applied were recovered. The peak InsP_4 kinase fractions that contained no significant InsP_4 phosphatase activity were pooled, 'spiked' with 1 mg of BSA and DTT (5 mM final concn.), dialysed against [50 mM-Hepes/2 mM-EGTA/15 mM 2-mercaptoethanol (pH 7.0 at 4 °C)]/glycerol (1:1, v/v) and stored at -20°C . These results were typical of those obtained in three independent preparations.

Subcellular and tissue distribution of InsP_4 kinase

^{32}P - and ^3H -labelled L- $\text{Ins}(1,4,5,6)\text{P}_4$ preparations from chick erythrocytes were used to assay the putative InsP_4 kinase. Activity was predominantly located in the supernatant fraction of macrophage homogenates. It was not diminished by dialysis, but was inactivated by heating at 90°C for 5 min or by omitting ATP from the incubation medium (see Table 1). InsP_4 kinase activity was also present in the supernatant fractions obtained from rat brain, parotid gland, heart and liver (Table 2). Little activity was found in the particulate fractions of each of the tissues assayed. The specific activity was greatest in the soluble fraction obtained from rat brain (approx. 8 times that in the liver supernatant).

Partial purification of InsP_4 kinase from rat brain cytosol

In order to identify the InsP_5 produced by the action of this kinase(s), an effort was made to produce a

partially purified and stable preparation of the activity which possessed sufficiently low InsP_4 phosphatase and ATPase activities to allow a substantial quantity of $[\text{^3H}]\text{InsP}_4$ to be phosphorylated in the presence of high-specific-radioactivity $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Rat brain cytosol was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation at 0°C . Each fraction was redissolved and dialysed to remove the $(\text{NH}_4)_2\text{SO}_4$ (see the Materials and methods section for further details) and the fractions were assayed for InsP_4 kinase, total ATPase, InsP_4 phosphatase and protein. In all, 40 % of the kinase activity was recovered in each of the 30–40 %- and 40–50 %-satd. $(\text{NH}_4)_2\text{SO}_4$ fractions, although the relative specific activity of the kinase compared with the InsP_4 phosphatase was 2-fold greater in the 40–50 %-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate. Consequently, as subsequent steps were essentially low-capacity purification processes, a 40–50 %-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate was used as a first step in the production of a partially purified InsP_4 kinase preparation.

Table 3. Partial purification of rat brain InsP_4 kinase activity

Results are means for duplicate determinations in units of d.p.m. converted/10 min per mg of protein for the InsP_4 kinase, InsP_4 phosphatase and InsP_5 phosphatase activities, and in nmol/min per μg of protein for total ATPase activity. A maximum of 30% of the available InsP_4 substrate was phosphorylated, and a maximum of 5% of the total InsP_4 substrate was dephosphorylated.

Enzyme assayed	Fraction . . .	Activity/fraction			
		Homogenate	Soluble	40–50 %-satd. (NH_4) ₂ SO_4	Mono-Q peak
InsP_4 kinase		1466	4511	15789	157980
InsP_4 phosphatase		80	201	548	4
Total ATPase		0.22	0.1	0.17	0.004
InsP_5 phosphatase		32	5	—	—

A sample of the 40–50 %-satd.-(NH_4)₂ SO_4 fraction that had been dialysed to remove (NH_4)₂ SO_4 was applied to a Mono-Q anion-exchange f.p.l.c. column and was eluted with a NaCl gradient (as described above). Each fraction was assayed for InsP_4 kinase, InsP_4 phosphatase and D- $\text{Ins}(1,4,5)\text{P}_3$ kinase activities.

The profile of InsP_4 kinase activity was clearly different from that of D- $\text{Ins}(1,4,5)\text{P}_3$ kinase and InsP_4 phosphatase (Fig. 2). The fractions containing InsP_4 kinase, but no InsP_4 phosphatase activity, were pooled and 'spiked' with recrystallized BSA and DTT. This combined fraction was dialysed against a glycerol-based buffer and stored at -20°C (see the legend to Fig. 2). This stored fraction contained an InsP_4 kinase specific activity 120 times greater than that of the parent homogenate, with greatly diminished ATPase, InsP_4 phosphatase and InsP_5 phosphatase activity (see Table 3).

The partially purified InsP_4 kinase converted [^3H]- InsP_4 from chick erythrocytes into a highly polar [^3H]Ins metabolite that co-chromatographed precisely with $\text{Ins}[^{32}\text{P}](1,3,4,5,6)\text{P}_5$ on a Partisil 10-SAX h.p.l.c. column (results not shown). The progress of this reaction with time is shown in Table 4. Approx. 70% of this

preparation of [^3H]Ins P_4 was rapidly converted into [^3H]Ins P_5 . The residual radioactivity was resistant to further additions of the kinase preparation (result not shown) and hence would not appear to be a substrate for the reaction. Periodate oxidation of this same preparation of [^3H]Ins P_4 revealed that 30% of it was periodate-resistant and that 70% of it was oxidized to yield [^3H]iditol upon reduction and dephosphorylation (results not shown). This [^3H]iditol can only be derived from L- $\text{Ins}(1,4,5,6)\text{P}_4$ (Stephens *et al.*, 1988). Thus this result confirms our interpretation above that the kinase converts L- $\text{Ins}(1,4,5,6)\text{P}_4$ into InsP_5 and that any other, periodate-insensitive, InsP_4 species in the preparation are apparently not substrates.

The native M_r of the rat brain InsP_4 kinase was determined by gel filtration on a Superose-12 gel-filtration f.p.l.c. column (see the Materials and methods section). A single peak of activity, containing 81% of the activity applied, was eluted 6.5 ml after V_0 (Fig. 3). Comparison with M_r standards suggest an M_r of 29000.

Identification of the product of partially purified InsP_4 kinase

By using the partially purified preparation of rat brain InsP_4 kinase to phosphorylate a portion of [^3H]Ins P_4 in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP, it was possible to incorporate ^{32}P into InsP_5 . This ^{32}P must be located at the site of the hydroxy group phosphorylated by InsP_4 kinase, i.e. at the L-3 substitution position (D-1 substitution position) or at the 2-position. The [^3H]Ins $^{[32}\text{P}]\text{P}_5$ produced in this way was purified by two h.p.l.c. steps, desalted and converted into its ammonium salt. Samples of this product co-chromatographed precisely with a relatively large quantity of [^3H]Ins(1,3,4,5,6) P_5 on a Partisil 10-SAX h.p.l.c. column and contained no $\text{Ins}^{[32}\text{P}]\text{P}$ species (results not shown). The position of the radioactive phosphate moiety was established by partial dephosphorylation with aq. NH_3 (see the Materials and methods section). This reaction randomly eliminates monoester phosphate groups from the inositol ring without allowing phosphate migration to occur (Tomlinson & Ballou, 1961), hence an $\text{Ins}^{[32}\text{P}]\text{P}$ species produced in this way will contain the phosphate inserted by InsP_4 kinase in its original position. The NH_3 hydrolysate was applied to a Partisil 5-PAC h.p.l.c. column (capable of resolving D/L- $\text{Ins}1\text{P}$ from $\text{Ins}2\text{P}$; see the Materials and methods section) with a mixture of D/L- $^{[14}\text{C}]\text{-Ins}1\text{P}$ and $^{[14}\text{C}]\text{-Ins}2\text{P}$ (see Fig. 4). The [^3H]Ins $^{[32}\text{P}]\text{P}$

Table 4. Phosphorylation to completion of the [^3H]Ins P_4 preparation used as a substrate for partially purified InsP_4 kinase

A total of 2000 d.p.m. of chicken erythrocyte [^3H]Ins P_4 was included in each assay (containing 200 μl of assay buffer and 7 μg of rat protein from the partially purified kinase preparation; for further details of the assay conditions, see the Materials and methods section). Each value in the Table is the mean for duplicate determinations, and similar results were obtained in one further experiment.

Time (min)	Proportion of total radioactivity in assay mixture (%)		
	Ins + Ins P + Ins P_2 + Ins P_3	Ins P_4	Ins P_5
0	3	96	0.5
5.5	1	68	31
10	1	51	48
15	3	37	60
20	1	34	65
30	3	30	67

Fig. 3. Gel filtration of rat brain cytosol InsP_4 kinase

Protein (0.9 mg) from a 40–50%-(NH_4) $_2\text{SO}_4$ fraction of rat brain cytosol dissolved in 150 μl of 0.5 M-KCl/50 mM-Hepes/2 mM-EGTA/15 mM-2-mercaptoethanol, pH 7.0 at 4 $^\circ\text{C}$, was pumped on to a fully equilibrated Superose-12 gel-filtration f.p.l.c. column at a rate of 0.3 $\text{ml}\cdot\text{min}^{-1}$. The A_{280} of the eluate was monitored continuously (—) and fractions were collected every 0.67 min from the V_0 (7.75 ml) through to V_t (18.8 ml). A 10 μl portion of each fraction was assayed for InsP_4 kinase activity (O) as described in the Materials and methods section. A maximum of 15% of the available substrate was converted into [^3H] InsP_5 . A maximum of 5% of the total substrate was dephosphorylated. In all, 81% of the InsP_4 kinase activity that was applied was recovered. The column was calibrated with M_r standards, which were independently eluted under identical conditions. Their elution positions are marked ($10^{-3} \times M_r$ values): 12.4, cytochrome *a*; 29, carbonic anhydrase; 45, ovalbumin; 66, BSA; 150, alcohol dehydrogenase. The elution position of the InsP_4 kinase activity suggests a native M_r of ~ 29000 .

co-chromatographed with D/L-[^{14}C] Ins1P . The product of the InsP_4 kinase reaction is therefore D- $\text{Ins}[\text{1-}^{32}\text{P}]\text{1,3,4,5,6P}_5$ [note that the insertion of [^{32}P] P_i eliminates the plane of symmetry in the otherwise *meso* compound $\text{Ins}(1,3,4,5,6)\text{P}_5$].

General discussion

In the preceding paper (Stephens *et al.*, 1988) we reported evidence for the presence of L- $\text{Ins}(1,4,5,6)\text{P}_4$ in mammalian and avian cells. In unstimulated macrophages it is the major species of [^3H] InsP_4 that can be detected, but, unlike D- $\text{Ins}(1,3,4,5)\text{P}_4$, its levels are insensitive to brief hormonal stimulation. The present results suggest that L- $\text{Ins}(1,4,5,6)\text{P}_4$ is a substrate for an enzyme that synthesizes a highly polar [^3H] Ins metabolite. On the basis of co-chromatography with radiochemically pure standards on Partisil 10-SAX anion-exchange resin, the polar metabolite made in macrophage homogenates is an InsP_5 species. The catalytic agent in macrophage homogenates that is apparently responsible for synthesis of InsP_5 is an ATP-requiring kinase that is found in the

Fig. 4. Separation of the $\text{Ins}[\text{32P}]\text{P}_5$ preparation on a Partisil 5-PAC h.p.l.c. column after digestion with aq. NH_3

After 100 h in boiling aq. NH_3 the $\text{Ins}[\text{32P}]\text{P}_5$ preparation was freeze-dried and redissolved in 1 ml of 20 mM-EDTA, pH 7.0. An aliquot of the hydrolysate (110000 d.p.m.) was mixed with D/L-[^{14}C] Ins1P and [^{14}C] Ins2P (prepared as described in the Materials and methods section) and applied to a pre-conditioned Partisil 5-PAC h.p.l.c. column. Fractions (0.4 ml) were collected (see the Materials and methods section). The earlier-eluted ^{14}C peak corresponds to D/L- Ins1P , the later-eluted one corresponding to Ins2P (see the Materials and methods section). The only ^{32}P -labelled compound (O) that was eluted under these conditions co-migrated with D/L-[^{14}C]- Ins1P (●). It represented 0.9% of the ^{32}P radioactivity applied.

cytosol and is highly selective for its substrate. D- $\text{Ins}(1,3,4,5)\text{P}_4$ will not serve as a substrate. This enzyme appears to be expressed *in vivo* as macrophages pre-labelled with [^3H] Ins contain [^3H] InsP_5 .

InsP_4 kinase is widely distributed among different tissues in the rat, including brain, heart, parotid and liver. In all of these tissues the activity is substantially soluble. The partially purified enzyme from rat brain cytosol is very different from the D- $\text{Ins}(1,4,5)\text{P}_3$ kinase found in the same tissue fraction (Irvine *et al.*, 1986; Morris *et al.*, 1988), both on the basis of its retention time on anion-exchange resin and its native M_r [D- $\text{Ins}(1,4,5)\text{P}_3$ kinase has a M_r of 200000, L- $\text{Ins}(1,4,5,6)\text{P}_4$ kinase one of 29000]. The product of the InsP_4 kinase when incubated in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP is D- $\text{Ins}[\text{1-}^{32}\text{P}]\text{1,3,4,5,6P}_5$, and hence the enzyme catalyses the transfer of the γ -phosphate group of ATP to the L-3 (D-1) hydroxy group of L- $\text{Ins}(1,4,5,6)\text{P}_4$.

L-Ins(1,4,5,6) P_4 is thus an intermediate in the production of an Ins P_5 species in mammalian and avian cells, the lack of a D-1 phosphate on this compound clearly distinguishing it from the inositol phosphates produced by phosphodiesteratic cleavage of any currently recognized inositol phospholipid. This implies that an independent series of inositol phosphate kinases might exist catalysing the reactions leading to the synthesis of Ins P_5 .

Chakrabarti & Majumder (1978) have presented evidence that an enzyme activity in avian erythrocytes and germinating seeds can sequentially phosphorylate Ins P through to Ins P_5 . The inositol phosphates used in these experiments were chemically synthesized and of mixed isomerism, hence the precise compounds utilized at each step have not been identified. Consequently whether or not any of the metabolites of D-Ins(1,4,5) P_3 can serve as substrates in this lineage remains unknown. If the synthesis of Ins P_5 is completely independent of the hormone-sensitive pathways of inositol-phospholipid degradation, then only two possible sources remain: either direct phosphorylation of inositol, or from glucose 6-phosphate via the reaction catalysed by inositol 1-phosphate synthase. A number of reports of inositol kinase activity have been published, although some workers have not been able to repeat the results of certain of these experiments (see Cosgrove, 1980). The strongest evidence in favour of such an enzyme is found in investigations of various plant tissues (e.g. English *et al.*, 1966), where the product has been shown to be L-Ins1 P (Loewus *et al.*, 1982). Inositol 1-phosphate synthase has been reported in both plant and animal tissues (Cosgrove, 1980); the product is L-Ins1 P in all cases. Although this reaction is the probable source of the inositol monophosphate precursor of phytate in angiosperms (Biswas *et al.*, 1978), it may not be the source of the production of Ins P_5 in avian erythrocytes, since Isaacks & Harkness (1976) have shown that, although [^{14}C]Ins could act as a precursor for Ins P_5 synthesis in chicken erythrocytes, [^{14}C]glucose could not. Resolution of these issues must await definitive identification of further precursors in the synthetic pathway to Ins P_5 .

In avian and amphibian erythrocytes, Ins P_5 is known to reduce the oxygen affinity of haemoglobin (Bartlett, 1980) in much the same fashion as 2,3-bisphosphoglycerate in mature human erythrocytes. Koppitz *et al.* (1986) have recently demonstrated the ability of micromolar concentrations of various inositol phosphates, including Ins P_5 , to inhibit aldolase A. Since Ins P_5 was not the most potent inhibitor of aldolase A among the inositol phosphates tested, and since haemoglobin is not

present in most mammalian cells, the general function of this compound remains unknown.

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