Product–precursor relationships amongst inositol polyphosphates

Incorporation of [^{32}P]P_i into *myo*-inositol 1,3,4,6-tetrakisphosphate, *myo*-inositol 1,3,4,5-tetrakisphosphate, *myo*-inositol 3,4,5,6-tetrakisphosphate and *myo*-inositol 1,3,4,5,6-pentakisphosphate in intact avian erythrocytes

Leonard R. STEPHENS* and C. Peter DOWNES†

Smith Kline and French Research Ltd., The Frythe, Welwyn, Herts. AL6 9AR, U.K.

Avian erythrocytes were incubated with *myo*-[^3H]inositol for 6–7 h and with [^{32}P]P_i for the final 50–90 min of this period. An acid extract was prepared from the prelabelled erythrocytes, and the specific radioactivities of the γ -phosphate of ATP and of both the *myo*-inositol moieties (^3H , d.p.m./nmol) and the individual phosphate groups (^{32}P , d.p.m./nmol) of [^3H]Ins[^{32}P](1,3,4,6)P₄, [^3H]Ins[^{32}P](1,3,4,5)P₄, [^3H]Ins[^{32}P](3,4,5,6)P₄ and [^3H]Ins[^{32}P](1,3,4,5,6)P₅ were determined. The results provide direct confirmation that one of the cellular InsP₄ isomers is Ins(1,3,4,5)P₄ which is synthesized by sequential phosphorylation of the 1,4,5 and 3 substitution sites of the *myo*-Ins moiety, precisely as previously deduced [Batty, Nahorski & Irvine (1985) *Biochem. J.* **232**, 211–215; Irvine, Letcher, Heslop & Berridge (1986) *Nature (London)* **320**, 631–634]. This is compatible with the proposed synthetic route from PtdIns via PtdIns4P, PtdIns(4,5)P₂ and Ins(1,4,5)P₃. The data also suggest that, in avian erythrocytes, the principle precursor of Ins(1,3,4,5,6)P₅ is Ins(3,4,5,6)P₄. Furthermore, if the γ - (and/or β -) phosphate of ATP is the precursor of the phosphate moieties of Ins(3,4,5,6)P₄, then this isomer must be derived from the phosphorylation of Ins(3,4,6)P₃. If the γ - (and/or β -) phosphate of ATP similarly acts as the ultimate precursor to all of the phosphates of Ins(1,3,4,6)P₄, then, in intact avian erythrocytes, the main precursor of Ins(1,3,4,6)P₄ is Ins(1,4,6)P₃. This contrasts with the expectation, based on results with cell-free systems, that Ins(1,3,4,6)P₄ is synthesized by the direct phosphorylation of Ins(1,3,4)P₃.

INTRODUCTION

Three different InsP₄ isomers have been identified in acid extracts of animal cells: D- or L-Ins(1,3,4,5)P₄ (Batty *et al.*, 1985), Ins(1,3,4,6)P₄ (Stephens *et al.*, 1988c) and Ins(3,4,5,6)P₄ (Stephens *et al.*, 1988a). Cellular Ins(1,3,4,5)P₄ is thought, on the basis of results obtained in cell-free assays, to be synthesized from Ins(1,4,5)P₃ [unambiguous evidence for the presence of Ins(1,4,5)P₃ in cells has recently been obtained (Stephens *et al.*, 1989) by use of an Ins(1,4,5)P₃ 3-hydroxykinase activity (Irvine *et al.*, 1986)].

Ins(1,3,4,6)P₄ was originally identified as the product of the phosphorylation of Ins(1,3,4)P₃ in rat liver homogenates (Shears *et al.*, 1987). This activity has since been described in homogenates or lysates derived from a number of other cell types (Balla *et al.*, 1987; Stephens *et al.*, 1988c). The presence of Ins(1,3,4,6)P₄ in cells has been assumed to be a result of the action of an Ins(1,3,4)P₃ 6-hydroxykinase activity upon cellular Ins(1,3,4)P₃ (Stephens *et al.*, 1988c).

The cellular origin of Ins(3,4,5,6)P₄ is currently undefined. Experiments designed to detect phosphorylation of a cell-derived [^3H]InsP₃ to [^3H]Ins(3,4,5,6)P₄ *in vitro* have repeatedly failed because insufficient product is formed to assign its structure (L. Stephens, unpublished work).

Two of the three cellular InsP₄ species defined above, Ins(1,3,4,6)P₄ and Ins(3,4,5,6)P₄, can act as precursors of Ins(1,3,4,5,6)P₅ in cell-free assays (Stephens *et al.*, 1988b,c). The soluble, ATP-dependent, chromatographically distinct Ins(1,3,4,6)P₄ 5-hydroxykinase and Ins(3,4,5,6)P₄ 1-hydroxykinase activities responsible can be detected in homogeneous populations of cells, e.g. avian erythrocytes or primary cultured macrophages (Stephens *et al.*, 1988c). Hence several questions related to the synthesis of InsP₅ have emerged. First, what is the metabolic origin of Ins(3,4,5,6)P₄ (in the light of the difficulties mentioned above), and secondly, on a more general note, are the pathways that can synthesize Ins(1,3,4,5)P₄, Ins(1,3,4,6)P₄, Ins(3,4,5,6)P₄ and Ins(1,3,4,5,6)P₅ *in vitro* active in intact cells? If so, what are their relative rates?

The experiments reported in this paper were aimed at tackling these problems by analysing the incorporation of [^{32}P]P_i tracer into the individual phosphate moieties of Ins(1,3,4,6)P₄, Ins(1,3,4,5)P₄, Ins(3,4,5,6)P₄ and Ins(1,3,4,5,6)P₅ in intact avian erythrocytes. They confirm expectations that cellular Ins(1,3,4,5)P₄ is synthesized from Ins(1,4,5)P₃. However, the data also suggest that, in intact cells, (1) Ins(1,3,4,6)P₄ is principally derived from Ins(1,4,6)P₃, (2) Ins(3,4,5,6)P₄ is derived from Ins(3,4,6)P₃ and (3) the major precursor of Ins(1,3,4,5,6)P₅ is Ins(3,4,5,6)P₄.

Abbreviations used: SAX, strong-anion-exchange; WAX, weak anion-exchange; BSA, bovine serum albumin.

* To whom correspondence and reprint requests should be sent, at present address: Department of Biochemistry, A.F.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

† Present address: Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, U.K.

MATERIALS AND METHODS

Erythrocytes from 5-day-old chicks were washed twice either in a Hepes-based medium [prepared as described previously (King *et al.*, 1987) except that it contained 0.38 mM- Na_2HPO_4], or in a NaCl-based medium (composition identical to that described above, except that the total Hepes concentration was reduced to 25 mM and replaced with 140 mM-NaCl) before being resuspended finally in the medium in which they had been washed at a density of 4 ml of packed erythrocytes/10 ml of total incubation volume. The erythrocyte suspensions were shaken in a thermostated water bath at 37 °C. Radioactive tracers that were to be introduced into the cell suspension were freeze-dried and dissolved in the incubation medium or in the final cell suspension.

Extraction and purification of [^3H]Ins[^{32}P]P species

Incubations were terminated by removing aliquots of the cell suspension (typically 250–500 μl). The cells were lysed and then acid-precipitated as described previously (Stephens *et al.*, 1988a). The acid extracts were treated with charcoal, neutralized (with KOH/Hepes), applied to a Partisil 10 strong-anion-exchange (SAX) column (90–95 % of exogenous [^3H]Ins P_4 and [^3H]Ins P_5 standards added to the original lysates were recovered at this point), and eluted as described in Stephens *et al.* (1988a). Fractions containing the Ins[^{32}P]P $_4$ s were pooled, as were those containing Ins[^{32}P]P $_5$. The pooled fractions were desalted (Stephens *et al.*, 1988c) and redissolved in 1 ml of 5.0 mM-EDTA (pH 7.0 with NaOH). These Partisil 10-SAX h.p.l.c.-purified [^3H]Ins[^{32}P]P $_4$ and [^3H]Ins[^{32}P]P $_5$ fractions were further purified by chromatography on a Partisphere weak-anion-exchange (WAX) h.p.l.c. column. [^3H]Ins[^{32}P](1,3,4,6)P $_4$, [^3H]Ins[^{32}P](1,3,4,5)P $_4$ and [^3H]Ins[^{32}P](3,4,5,6)P $_4$ were resolved as described previously (Stephens *et al.*, 1988c; this chromatographic system could reliably resolve the InsP $_4$ s from up to 0.3 ml of packed avian erythrocytes). Vials containing a single [^3H]Ins[^{32}P]P $_4$ isomer were pooled and desalted as described above. Samples of [^3H]Ins[^{32}P](1,3,4,6)P $_4$ to be selectively dephosphorylated (see below) were further purified by one additional cycle of chromatography through a Partisphere WAX column. [^3H]Ins[^{32}P](3,4,5,6)P $_4$ similarly destined for selective dephosphorylation was repurified by one or two additional cycles of chromatography on a Partisphere WAX h.p.l.c. column until 99.5 % radiochemically pure. [^3H]Ins[^{32}P]P $_5$ was repurified on a Partisphere WAX h.p.l.c. column which was eluted as described for the [^3H]Ins[^{32}P]P $_4$ species above, but with the following gradient: 0 min, 0 % B; 5 min, 0 % B; 65 min, 100 % B; 70 min, 100 % B; 71 min, 0 % B [B = 0.5 M-(NH_4) $_2\text{HPO}_4$ /H $_3\text{PO}_4$, pH 3.2, 25 °C]. The fractions containing [^3H]Ins[^{32}P]P $_5$ were pooled and desalted as described.

Extraction of ATP

ATP was extracted from 50 μl aliquots of erythrocyte suspensions (see above) and the specific radioactivity of its γ -phosphate was determined as described by King *et al.* (1987). The concentration of ATP in the extract was measured with an enzyme-linked bioluminescence assay (Chrono-log Corp.). Sample luminescence was determined with a Chrono-log aggrrometer.

Dual-label liquid scintillation counting

Aqueous samples containing ^3H and/or ^{32}P radioactivity were counted in a Beckman benchtop LS 1801 liquid scintillation counter utilizing the associated dual-label, decay and quench-correction software. A range of chloroform-quenched ^3H and ^{32}P standards were counted (under conditions identical with those used to count all experimental samples; see below) to calibrate the scintillation counter. All radioactive solutions to be counted were dissolved in a scintillation fluid mixture of the following composition: 10 ml of Insta-gel (Packard), 2 ml of CH_3OH /water (1:1, v/v) and 0.4 ml of h.p.l.c. column eluant (0–100 % B). The salt content of the h.p.l.c. eluant significantly altered the quench environment of the samples, but this was corrected appropriately by the quench-correction procedure. (The system was tested by mixing defined aliquots of ^3H and ^{32}P with a series of fractions containing various percentages of B, then dissolving them in the scintillation fluid cocktail defined above and counting them for radioactivity with the calibrated counter described.) The values of the background ^3H and ^{32}P that were subtracted from radioactive peak totals were defined by blank vials run with every experiment.

Measurement of Ins

The concentrations and specific radioactivities of h.p.l.c.-purified [^3H]inositol phosphates were determined by assaying the Ins liberated after dephosphorylation (see below), essentially as described previously (MacGregor & Matschinsky, 1984) with certain procedural modifications as noted below. Inositol phosphates were dephosphorylated with alkaline phosphatase (Sigma, type P-5521). [^3H]InsP $_4$ species were treated as described previously (Stephens *et al.*, 1988a), except that the assay buffer contained 10 mM-triethanolamine, pH 8. [^3H]InsP $_5$ was incubated at 25 °C for 10 h in 2 ml of 10 mM-triethanolamine (pH 7.0 with HCl) containing 20 units of alkaline phosphatase/ml (ammonium-sulphate-free). The samples were desalted with 2 ml of MB 3 mixed-bed ion-exchange resin. A total of 92–100 % of the [^3H]Ins contained in the [^3H]InsP $_4$ s and [^3H]InsP $_5$ was recovered through this procedure. The Ins assay was performed essentially as described previously (MacGregor & Matschinsky, 1984). The samples were incubated with 0.3 units of *myo*-inositol dehydrogenase/ml (Sigma; the specific activities of the inositol dehydrogenase preparations were routinely tested, as described by Sigma, immediately before their inclusion in an assay) for 120 min. Under these conditions, up to 8 nmol of Ins could be quantitatively oxidized, yielding 8 nmol of NADH in the process. The fluorescence of the final samples was measured in a Perkin-Elmer LS-5 luminescence spectrophotometer. The total ^3H present in Ins assays was determined by counting the assay contents after completion of the fluorimetric measurements. The specific radioactivity of the [^3H]Ins moiety in the original [^3H]inositol phosphate was calculated directly from the total ^3H radioactivity and the total Ins present in the assay. The specific radioactivity of ^{32}P in a [^3H]Ins[^{32}P]P $_x$ was determined by measuring the specific radioactivity of the [^3H]Ins moiety (as described above), and hence, from a knowledge of the $^3\text{H}/^{32}\text{P}$ content of the original [^3H]Ins[^{32}P]P $_x$ (previously established by dual-label

liquid-scintillation counting), that of the ^{32}P could be calculated.

Phosphate assay of inositol phosphates

H.p.l.c.-purified, desalted $[\text{}^3\text{H}]\text{Ins}[\text{}^{32}\text{P}]\text{P}_x$ were also quantified by phosphate assay. ^{31}P n.m.r. analysis of inositol phosphate preparations, desalted by the methods described above, revealed that although they were essentially free from P_i , they contained significant quantities of PP_i (results not shown). As PP_i is hydrolysed during the 'work-up' phase of phosphate assays designed to quantify organic phosphates, a protocol was devised to remove PP_i from desalted inositol phosphate fractions. PP_i was eliminated at two stages. First, 95–96 % of total PP_i could be eluted from the desalting columns with 10 ml of 0.7 M-ammonium formate/0.1 M-formic acid ($[\text{}^{32}\text{P}]\text{PP}_i$ was used to trace the fate of PP_i during standard desalting protocols; results not shown) without significant loss of InsP_4 s or InsP_5 s. Secondly, PP_i could be removed by incubating the desalted preparations of inositol phosphates with inorganic PP_i phosphatase (Sigma). $[\text{}^3\text{H}]\text{Ins}[\text{}^{32}\text{P}]\text{P}_4$ s and $[\text{}^3\text{H}]\text{Ins}[\text{}^{32}\text{P}]\text{P}_5$ were incubated for 45 min in 1 ml of buffer containing 50 units of inorganic PP_i phosphatase/ml and 10 mM-Hepes, pH 7.2, at 25 °C (under these conditions, 0.5 mM- $[\text{}^{32}\text{P}]\text{PP}_i$ was converted completely to $[\text{}^{32}\text{P}]\text{P}_i$ without significant dephosphorylation of any of the $[\text{}^3\text{H}]\text{Ins}[\text{}^{32}\text{P}]\text{P}_4$ s or of $[\text{}^3\text{H}]\text{Ins}[\text{}^{32}\text{P}]\text{P}_5$). The reactions were quenched with 20 μl of 70 % (w/v) HClO_4 and neutralized with KOH, and P_i was removed by passing the samples through a standard desalting protocol (incorporating a wash with 0.7 M-ammonium formate/0.1 M-formic acid, see above). The desalted PP_i -free inositol phosphate preparations were finally assayed for total phosphorus [as described by Bartlett (1959) and modified by Galliard *et al.*, (1965)] and free inorganic phosphate (Baginski *et al.*, 1967). The quantity of InsP_4 or InsP_5 was calculated from the difference between the total and free phosphate in the preparations (the free phosphate concentration was always below the detection limit of the assay) and the assumption that the ' InsP_4 s' and ' InsP_5 s' possessed phosphorus/Ins ratios of 4 and 5 respectively. Fractions of h.p.l.c. eluant which neighboured those pooled to provide the inositol phosphate preparations were processed as described and served as overall blanks for the assay procedure.

Extraction and desalting of inositol phosphates from scintillation fluid/h.p.l.c. eluate mixtures

To maximize the precision with which the $^3\text{H}/^{32}\text{P}$ content of inositol phosphates could be measured and yet still allow them to be further metabolized or oxidized, it proved necessary to extract them from scintillation fluid/h.p.l.c. eluate mixtures. This was achieved by mixing the scintillation fluid/water/ CH_3OH /h.p.l.c. eluate (see under 'dual-label liquid scintillation counting') with 0.33 vol. of chloroform and 0.5 vol. of water. This solvent mixture separated into two clear phases upon centrifugation, with scintillants and organic solvents in the lower phase. Experience showed that addition of further phosphate buffer to this solvent system resulted in a more complete partitioning of the detergents into the lower phase; this effect was routinely exploited by adding sufficient 1.25 M- $(\text{NH}_4)_2\text{HPO}_4$ (pH 3.8, with H_3PO_4) to the system to raise the total phosphate concentration of the upper phase to approx. 30 mM. After centrifugation,

the upper phase was removed and its pH was adjusted to 6–6.5 with triethylamine. Samples that were to be desalted were diluted with water until the total concentration of phosphate had been reduced to levels that would allow the inositol phosphates contained in the fraction to bind to a 2 cm column of Bio-Rad AG 1.8 (200–400, in the formate form) anion-exchange resin. In practice, this meant that InsP_4 s which had been eluted in phosphate buffer from a Partisphere SAX h.p.l.c. column (see below) could be taken straight through all of the above steps with no additional phosphate or water being required. Fractions of Partisphere SAX h.p.l.c. eluate containing InsP_5 s and InsP_3 s (to which additional phosphate had been added to reduce detergent carry-over) required an additional 0.5 vol. and 0.16 vol. (relative to the original volume of scintillation fluid/water/ CH_3OH /h.p.l.c. eluate mixture) of water respectively to allow them to bind quantitatively to the desalting column. P_i and inositol phosphates were eluted from the desalting column with ammonium formate/formic acid mixtures as described previously (Stephens *et al.*, 1988c); the resulting solutions were lyophilized directly. Fractions of inositol phosphates extracted from scintillation fluid and desalted in this way were oxidized readily by sodium periodate and metabolized by various enzyme preparations (see below).

Preparation of human erythrocyte ghosts

Human erythrocyte ghosts were prepared essentially as described by Hawkins *et al.* (1984), except that the ghosts were washed with 20 mM-Tris-HCl/1 mM-EDTA (pH 7.2, 4 °C) until white and then with 2.5 mM-Hepes/1 mM-EGTA (pH 7.2, 4 °C) until all of the cytoskeletal material had been washed out and the ghosts had 'collapsed' to approx. 20 % of their original volume; they were stored in this condition at –70 °C until used.

Preparation of rat brain homogenates and soluble fractions

Rat brain homogenates and soluble fractions were freshly prepared in the presence of 0.1 mM-phenylmethanesulphonyl fluoride and antipain, pepstatin A and leupeptin (1 $\mu\text{g}/\text{ml}$ of each) as described previously (Stephens *et al.* 1988c). The homogenate and soluble fractions typically possessed protein concentrations of 11.0 and 2.5 mg/ml respectively.

Preparation of $\text{Ins}(1,3,4,6)\text{P}_4$ 6-phosphate, $\text{Ins}(1,3,4,6)\text{P}_4$ 3-phosphate and $\text{Ins}(1,3,4,6)\text{P}_4$ 4-phosphate phosphomonoesterase activities

Partially purified fractions of $\text{Ins}(1,3,4,6)\text{P}_4$ 6-phosphate phosphomonoesterase activity (native molecular mass of 150 kDa), $\text{Ins}(1,3,4,6)\text{P}_4$ 3-phosphate and $\text{Ins}(1,3,4,6)\text{P}_4$ 4-phosphate phosphomonoesterase activities (the latter two activities possess native molecular masses of 31 kDa and were not resolved) were purified from rat brain cytosol by gel-permeation chromatography, precisely as described previously (Stephens *et al.*, 1989). Typically, 1.7 mg of protein from a 20–50 % $(\text{NH}_4)_2\text{SO}_4$ (% saturation at 4 °C) fraction of rat brain cytosol was applied to a Superose-12 gel-filtration column (Pharmacia). Fractions of proteins with native molecular masses of 150 kDa and 31 kDa were pooled and used to dephosphorylate $\text{Ins}(1,3,4,6)\text{P}_4$ to defined InsP_3 s (see below).

Separation of inositol phosphate dephosphorylation products by anion-exchange h.p.l.c.

The mixtures of inositol phosphates produced during the enzyme-catalysed dephosphorylation of inositol phosphates (see below) were resolved on a Partisphere 5-SAX anion-exchange h.p.l.c. column (11.0 cm $\times$ 0.47 cm, Whatman) which was eluted at 1.0 ml $\cdot$ min⁻¹ with a gradient based on water (A) and 1.25 M-(NH₄)₂HPO₄ (B) (pH 3.8 with H₃PO₄, 25 °C), as follows: 0 min, 0 % B; 12 min, 0 % B; 25 min, 8 % B; 52 min, 17 % B; 53 min, 23 % B; 87 min, 23 % B; 107 min, 100 % B; 112 min, 100 % B; 113 min, 0 % B. Fractions were collected every 0.4 min and mixed with 2 ml of CH₃OH/water (1:1, v/v) and 10 ml of Insta-gel (see above).

Inositol monophosphates were resolved with a Partisphere 5-SAX anion-exchange h.p.l.c. column (see above) but were eluted at 1.0 ml $\cdot$ min⁻¹ with a gradient based on water (A) and 0.2 M-potassium acetate (B) (pH 3.75 with acetic acid, 25 °C) as follows: 0 min, 0 % B; 10 min, 0 % B; 12 min, 48 % B; 70 min, 48 % B; 71 min, 0 % B. If the sample being resolved on the above gradient contained some InsP₂s, then to elute these from the column it was necessary to switch buffer B (once the gradient described above had been completed) to 1.25 M-(NH₄)₂HPO₄ (pH 3.8 with H₃PO₄, 25 °C) and continue eluting the column at 25 % B.

Periodate oxidation of inositol phosphates

Scintillant-free and salt-free preparations of inositol phosphates were oxidized with sodium periodate (InsP₂s were oxidized with 0.1 M-periodic acid, pH 3.0 with NaOH), reduced and dephosphorylated as described previously (Stephens *et al.*, 1988a,c). The [³H]polyols recovered were separated on a polypore-carbohydrate cation-exchange h.p.l.c. column (in the Pb²⁺ mode, as described in Stephens *et al.*, 1988c, 1989). The optical isomerism of unknown [³H]iditols and [³H]altritols was determined by incubating the [³H]polyols with a yeast-derived preparation of L-iditol dehydrogenase (Sigma) in the presence of D-[¹⁴C]iditol or L-[¹⁴C]altritol standards precisely as described previously (Stephens *et al.*, 1989).

Dephosphorylation of [³H]Ins[³²P](1,3,4,5)P₄ by rat brain homogenates

[³H]Ins[³²P](1,3,4,5)P₄, purified from acid extracts of [³H]Ins- and [³²P]P_i-prelabelled avian erythrocytes, was incubated in 2 ml assays which contained 50 mM-Hepes/2 mM-EGTA/1 mM-MgCl₂/250 μ l of rat brain homogenate (see above)/10 mM-LiCl/0.28 μ M-[³H]Ins[³²P](1,3,4,5)P₄ (pH 7.0, 37 °C) for 0, 3 or 10 min. The reactions were quenched with 50 μ l of 70 % (w/v) HClO₄ and processed for h.p.l.c. on a Partisphere 5-SAX anion-exchange column as described above. Fractions (0.4 min) were collected and individually dual-label counted. Fractions containing single peaks of ³H and ³²P radioactivity were pooled together, and the inositol phosphates were extracted from the scintillation fluid in which they had been counted and desalted (as described above). Aliquots of each of the scintillant and salt-free inositol phosphate preparations were oxidized with sodium periodate and the [³H]polyols produced were identified as described above. This basic protocol of post-acid processing and chromatography followed by quantification and characterization of the reaction products was routinely applied after dephosphorylating other [³H]Ins[³²P]Ps (see below).

Dephosphorylation of [³H]Ins[³²P](1,3,4,6)P₄

Aliquots of [³H]Ins[³²P](1,3,4,6)P₄, which had been extracted and purified from acid extracts of [³H]Ins- and [³²P]P_i-prelabelled avian erythrocytes (as described above), were incubated for 0 or 40 min with a partially purified preparation of Ins(1,3,4,6)P₄ 6-phosphate phosphomonoesterase in 1.5 ml assays containing 50 mM-Hepes/2 mM-EGTA/1 mM-MgCl₂/166 mM-KCl (pH 7.0, 37 °C)/0.5 ml of gel-filtration column eluate containing the phosphomonoesterase activity [final concentration of Ins(1,3,4,6)P₄ was approx. 0.72 μ M]. Some of the same preparations of [³H]Ins[³²P](1,3,4,6)P₄ were incubated with partially purified Ins(1,3,4,6)P₄ 3-phosphate and 4-phosphate phosphomonoesterase activities (see above). The assays (9 ml total volume) contained 3 ml of gel-filtration column eluate (the same 3 $\times$ 0.5 ml fractions, centred around the elution volume expected for 31 kDa proteins, were pooled from two identical gel-filtration separations, each of which was performed precisely as described above) and 50 mM-Hepes/2 mM-EGTA/166 mM-KCl/1 mM-MgCl₂ (pH 7.0, 37 °C); the final concentration of [³H]Ins[³²P](1,3,4,6)P₄ was approx. 0.12 μ M. Both types of assay were quenched with HClO₄, and the precipitated protein and HClO₄ were removed as described (Stephens *et al.*, 1988a).

The samples were diluted 5-fold with water and applied to a 2 cm column of Bio-Rad AG 1X8 (200–400 in the formate form) anion-exchange resin. Of the chloride applied to the columns, 98–99 % was eluted with 20 ml of 0.2 M-ammonium formate/0.1 M-formic acid. (³⁶Cl⁻ was used to trace the movement of chloride in a series of trial separations. The quantity of chloride in the assay buffer used above was sufficient to reduce the quality of the separation obtained during the subsequent h.p.l.c. step; consequently, samples were routinely desalted as described.) [³H]Ins[³²P]P₃s and residual [³H]Ins[³²P]P₄ were eluted and desalted (Stephens *et al.*, 1988c). The desalted [³H]Ins[³²P]P₃s and residual [³H]Ins[³²P]P₄ were dissolved in 5 mM-EDTA (pH 7.0 with NaOH) and applied to a Partisphere 5-SAX h.p.l.c. column which was eluted, and the resolved products were quantified and characterized as described above.

A portion of the [³H]Ins[³²P]P₃ produced from [³H]Ins[³²P](1,3,4,6)P₄ by the 150 kDa Ins(1,3,4,6)P₄ 6-phosphate phosphomonoesterase [i.e. [³H]Ins[³²P](1,3,4)P₃] was incubated with a rat brain cytosol fraction for 0 or 6 min in 2 ml of buffer containing 50 mM-Hepes/2 mM-EGTA/1.0 mM-LiCl/50 μ l of rat brain cytosol fraction (prepared as described above), pH 7.0 at 37 °C. The reactions were quenched with 50 μ l of 70 % (w/v) HClO₄, the incubations were processed and the products were chromatographed, quantified and characterized as described above.

Dephosphorylation of [³H]Ins[³²P](3,4,5,6)P₄

Aliquots of purified [³H]Ins[³²P](3,4,5,6)P₄ were incubated for 0 or 100 min with human erythrocyte ghosts (prepared as described above) in a 6.8 ml assay containing 1.35 ml of packed human erythrocyte ghosts, 1 mg of bovine serum albumin (BSA)/ml, 4 mM-MgCl₂, 7 mM-Hepes and 2 mM-EGTA, pH 7.0 (37 °C). The reactions were quenched with 135 μ l of 70 % (w/v) HClO₄ and diluted to 17 ml with water. The precipitated debris was pelleted in an ultracentrifuge (100 000 *g* for 7 min; 4 °C) and the supernatant was neutralized with 2 M-KOH/

50 mM-Hepes. The samples were mixed with 400 μ l of 0.1 M-EDTA (pH 7.0) and reduced in volume to 2.5 ml by freeze-drying. The KClO_4 was pelleted in a benchtop centrifuge and the reaction products were chromatographed, quantified and characterized as described above.

A portion of the major $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_2$, [i.e. $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4)P_2$] produced during the dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ was incubated with a rat brain cytosol fraction for 0 or 30 min in 1.4 ml of buffer containing 5 mM-EDTA/25 mM-Hepes/1 mM-EGTA (pH 7.0)/250 μ l of rat brain cytosol (prepared as described above) at 37 °C. The reactions were quenched with 35 μ l of 70 % (w/v) HClO_4 and processed as described in Stephens *et al.* (1988a). The HClO_4 -free supernatant was applied to a Partisphere 5-SAX h.p.l.c. column and eluted into 0.4 min fractions as described. All of the fractions were individually counted for ^{32}P and ^3H radioactivity as described above. The peak of $[^3\text{H}]\text{Ins}[^{32}\text{P}]P$ resolved from the dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ was extracted from the scintillant in which it had been counted, diluted 20-fold with water and neutralized with triethylamine. After mixing with $[^{14}\text{C}]\text{Ins}3P$, $[^{14}\text{C}]\text{Ins}2P$ and $[^{14}\text{C}]\text{Ins}4P$ (prepared as described by Stephens *et al.*, 1988c), the sample was applied to a Partisphere 5-SAX h.p.l.c. column and eluted with an acetate-based buffer as described above. Fractions were collected every 0.5 min and counted individually for ^{14}C , ^3H and ^{32}P radioactivity utilizing standard triple-label liquid scintillation counting techniques.

Dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$

Aliquots of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$, which had been purified from acid extracts of $[^3\text{H}]\text{Ins}/[^{32}\text{P}]P_i$ -prelabelled avian erythrocytes (as described above) were incubated with human erythrocyte ghosts for 0 or 180 min. The incubation (total volume 38 ml) contained 7.28 ml of packed erythrocyte ghosts, 1 mg of recrystallized BSA/ml, 1 mM- MgCl_2 , 5 mM-Hepes and 1 mM-EGTA (pH 7.0, 37 °C). The final concentration of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ was approx. 4.0 μM . Reactions were quenched with 766 μ l of 70 % (w/v) HClO_4 and then processed for chromatography, and the products were quantified and subsequently identified as described for $\text{Ins}(3,4,5,6)P_4$ above.

Portions of the scintillant- and salt-free preparations of the major $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_2$ dephosphorylation product of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ [i.e. $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4)P_2$] were incubated with rat brain cytosol. The assays were run for 0 or 4 min in 2 ml of buffer containing 2 mM-EGTA, 1 mM- MgCl_2 , 6.45 mM-Hepes (pH 7.0, 37 °C) and 2.0 mM of the potassium salt of DL- $\text{Ins}1P$ (Sigma). This concentration of DL- $\text{Ins}1P$ reduced the rate of $[^3\text{H}]\text{Ins}[^{32}\text{P}]4P$ hydrolysis sufficiently to allow complete metabolism of the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4)P_2$ without a significant accumulation of free $[^3\text{H}]\text{Ins}$. Reactions were quenched with 40 μ l of 70 % (w/v) HClO_4 , treated with tri-*N*-octylamine/Freon (1:1, v/v), mixed with 40 μ l of 0.1 M-EDTA (pH 7.0) and $[^{14}\text{C}]\text{Ins}3P$, and applied to a Partisphere 5-SAX h.p.l.c. column. The column was eluted with an acetate-based gradient and then with a phosphate-based gradient (see above). Fractions of 0.4 min were collected and counted twice, first for ^3H and ^{32}P with the dual-label program described above, and secondly with a triple-label (^3H , ^{14}C and ^{32}P) program (as all of the ^{14}C eluted in fractions which contained no ^3H

Fig. 1. Concentration of ATP, and the incorporation of $[^{32}\text{P}]P_i$ into the γ -phosphate of ATP, in avian erythrocytes incubated *in vitro*

Avian erythrocytes were prepared as described in the Materials and methods section and washed twice in either an Na Hepes-based (●, ■) or an NaCl-based (○, □) medium, then shaken at 37 °C with $[^{32}\text{P}]P_i$ (100 $\mu\text{Ci}/\text{ml}$, final) for various times before the addition of ice-cold 5 % (v/v) HClO_4 . After vigorous mixing and 5 min standing on ice, the insoluble material was pelleted by centrifugation and the supernatant was neutralized with tri-*N*-octylamine/Freon (1:1, v/v; see the Materials and methods section). The concentration of the ATP (□, ■) and the specific radioactivity of its γ -phosphate (○, ●) were measured as described in the Materials and methods section. Data shown are means of independent duplicate assays from two separate experiments measuring the specific radioactivity of the γ -phosphate of ATP in avian erythrocytes incubated in Hepes-based media and a single experiment for the remaining data.

or ^{32}P radioactivity, the precision of the ^3H and ^{32}P counts was not compromised).

A portion of the salt-free and scintillant-free preparation of the third $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_3$ eluted from the h.p.l.c. column {derived from $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ } was incubated with human erythrocyte ghosts for a further 0, 70 or 120 min. The incubations (total vol. 2.0 ml) contained 2 mM- MgCl_2 , 1 mg of recrystallized BSA/ml, 500 μ l of packed human erythrocyte ghosts, 12 mM-Hepes and 1 mM-EGTA (pH 7.0, 37 °C). The assays were quenched with 40 μ l of 70 % (w/v) HClO_4 , and then were processed, chromatographed and the products resolved, quantified and then fully characterized as described above.

Radiochemicals

$[^3\text{H}]\text{Ins}$ was purchased from New England Nuclear. $[^{32}\text{P}]P_i$ (PBS-13), $^{36}\text{Cl}^-$ and $[^{32}\text{P}]\text{PP}_i$ were purchased from Amersham International.

Materials

Partisphere SAX and WAX h.p.l.c. columns (0.46 cm $\times$ 12.5 cm) were obtained from Whatman. Poly-pore Pb^{2+} h.p.l.c. columns were obtained from Anachem.

RESULTS

Incorporation of ^{32}P into ATP, $\text{Ins}P_4$ s and $\text{Ins}P_5$ in avian erythrocytes incubated *in vitro*

When avian erythrocytes were washed and incubated in a chloride-free Hepes-based medium (Fig. 1), they

Table 1. Incorporation of [^3H]Ins into InsP_4 s and InsP_5 in avian erythrocytes incubated *in vitro*

Avian erythrocytes were prepared, incubated and their inositol phosphates extracted and purified exactly as described in the legend to Fig. 2. The total ^3H radioactivity associated with each inositol phosphate was quantified by dual-label scintillation counting. The data shown are from a single experiment; one further experiment yielded qualitatively identical data.

Inositol phosphate	Time of incubation (min)...	Total ^3H (d.p.m.)				
		15	50	100	200	425
$[^3\text{H}]\text{Ins}(1,3,4,6)\text{P}_4$		80	9408	87470	708700	4952512
$[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$		825	50858	410820	2667073	12136656
$[^3\text{H}]\text{Ins}(3,4,5,6)\text{P}_4$		5	2391	48036	613980	4635584
$[^3\text{H}]\text{Ins}(1,3,4,5,6)\text{P}_5$		60	17710	283955	2317849	13558867

Fig. 2. Incorporation of [^{32}P]P $_i$ into $\text{Ins}(1,3,4,6)\text{P}_4$, $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(3,4,5,6)\text{P}_4$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$ in avian erythrocytes incubated *in vitro*

A 0.6 ml portion of packed avian erythrocytes was incubated with 5 mCi of [^{32}P]P $_i$ in a total volume of 1.5 ml (the medium also contained 3.3 mCi of [^3H]Ins/ml; see Table 1). Aliquots (250 μl) of the suspension were removed at various times. Inositol phosphates were extracted and purified on a Partisil 10-SAX anion-exchange h.p.l.c. column to yield $\text{Ins}[^{32}\text{P}]\text{P}_4$ and $\text{Ins}[^{32}\text{P}]\text{P}_5$ fractions ($\circ$, $\text{Ins}[^{32}\text{P}](1,3,4,5,6)\text{P}_5$; $\bullet$, $\text{Ins}[^{32}\text{P}](1,3,4,5)\text{P}_4$; $\blacksquare$, $\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$; $\blacktriangle$, $\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$). The $\text{Ins}[^{32}\text{P}]\text{P}_4$ s were further fractionated on a Partisphere WAX h.p.l.c. column. The ^{32}P and ^3H contents (see also Table 1) of the various inositol phosphates were measured by dual-label liquid scintillation counting. The smaller quantity of cells used in this analysis (0.1 ml of packed cell volume/sample, compared with 0.2 ml of packed cell volume/sample for most of the remaining experiments) meant that the $\text{Ins}[^{32}\text{P}](1,3,4,5)\text{P}_4$ that spilled into the $\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ fractions never amounted to more than 10% of the total $\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ (results not shown). A curve describing the incorporation of [^{32}P]P $_i$ into the γ -phosphate of ATP, in cells incubated under identical conditions, is also shown (see Fig. 1). The data presented are from a single experiment; one further experiment generated qualitatively similar results. The time frame within which the [^{32}P]P $_i$ labelling experiments reported elsewhere in this paper were performed is indicated in the Figure.

maintained their ATP levels within the normal range, but the initial rate at which they incorporated [^{32}P]P $_i$ from the medium into the γ -phosphate of ATP was 9 times more rapid than if they were incubated in the presence of chloride. This phenomenon has been well documented in mammalian erythrocytes (Whittam, 1964; King *et al.*, 1987) and is a result of the fact that chloride competes more effectively than phosphate for the anion transporter in erythrocyte plasma membranes. Avian erythrocytes presumably exchange phosphate across their plasma membranes by an analogous mechanism.

Avian erythrocytes were incubated in a HEPES-based medium containing [^{32}P]P $_i$ and [^3H]Ins, and acid extracts of the cells were prepared, treated with charcoal, neutralized and resolved on an anion-exchange h.p.l.c. column (see the Materials and methods section for details). Two major peaks of ^{32}P eluted from the h.p.l.c. column after ATP. These peaks coincided precisely with the only two peaks of ^3H -labelled material in this region of the chromatographic profile and at the times expected for InsP_4 s and InsP_5 s. The fractions containing [^3H]Ins $^{32}\text{P}]\text{P}_5$ (s) were pooled, desalted and rechromatographed on a WAX h.p.l.c. column (see the Materials and methods section). The peak fractions were again pooled and desalted. The fractions containing the [^3H]Ins $^{32}\text{P}]\text{P}_4$ s were pooled, desalted and rechromatographed on a WAX column (see above). Three peaks of ^{32}P were eluted at the times expected for InsP_4 s (^3H radioactivity eluted with each of these peaks; results not shown). The first and third eluting peaks have been shown to be [^3H]Ins $^{32}\text{P}](1,3,4,6)\text{P}_4$ and [^3H]Ins $^{32}\text{P}](3,4,5,6)\text{P}_4$ respectively (Stephens *et al.* 1988c); the second eluting peak has been established to be [^3H]Ins $^{32}\text{P}](1,3,4,5)\text{P}_4$ (see below). All three [^3H]Ins $^{32}\text{P}]\text{P}_4$ s were isolated, desalted and rechromatographed, on the same type of h.p.l.c. column, a further one or two times depending on the purity of the [^3H]Ins $^{32}\text{P}]\text{P}_4$ preparations required.

The quantities of ^3H and ^{32}P contained in [^3H]Ins $^{32}\text{P}]\text{P}_4$ s and [^3H]Ins $^{32}\text{P}]\text{P}_5$ extracted and purified from avian erythrocytes which had been incubated for various times are shown in Table 1 and Fig. 2. Both tracers entered the pools of inositol polyphosphates with substantially 'lagged' progress curves, this being particularly marked for the incorporation of [^3H]Ins into

Table 2. Concentration and specific radioactivities of [³H]Ins[³²P]₄s and [³H]Ins[³²P]₅ in avian erythrocytes incubated *in vitro* with [³²P]P_i and [³H]Ins for 80 min

All samples were purified initially on a Partisil 10-SAX anion-exchange h.p.l.c. column, and all subsequent steps were on a Partisphere 5-WAX anion-exchange h.p.l.c. column. A 0.72 ml portion of packed avian erythrocytes was incubated with [³H]Ins (80 min, 2.7 mCi/ml) and [³²P]P_i (80 min, 2.7 mCi/ml) as described above. Aliquots (0.5 ml) of the suspension were removed, quenched and their inositol phosphates extracted and purified as described above. Aliquots (50 μl) of the suspension were withdrawn to assay the specific radioactivity of the γ-phosphate of ATP (as described in the Materials and methods section). The ³H/³²P d.p.m. ratios of the purified inositol phosphates were determined by dual-label liquid scintillation counting and the concentrations of Ins(3,4,5,6)P₄ and Ins(1,3,4,5,6)P₅ were measured by phosphate assay (as described in the Materials and methods section). The data presented are means ± S.E.M. (n = 3). The concentrations of Ins(1,3,4,6)P₄ and Ins(1,3,4,5)P₄ were too low, relative to the blanks, to be accurately measured by phosphate assay. In a parallel experiment (results not shown) the proportion of the total [³²P]P_i in the 1-phosphate of [³H]Ins[³²P](1,3,4,5,6)P₅ was 47% (see Tables 7 and 8 for a description of how this was achieved). The ³²P specific activity of the γ-phosphate of ATP was 100300 ± 1100 d.p.m./nmol.

Inositol phosphate	H.p.l.c. purification steps	³ H/ ³² P ratio	Cellular concn. (nmol/ml)	Specific radioactivity (d.p.m./nmol)	
				³ H	³² P
Ins(1,3,4,6)P ₄	2	1.44 ± 0.015	—	—	—
Ins(1,3,4,5)P ₄	2	1.89 ± 0.025	—	—	—
Ins(3,4,5,6)P ₄	3	0.80 ± 0.030	82.0 ± 8.5	1486 ± 163	1864 ± 163
Ins(1,3,4,5,6)P ₅	2	0.29 ± 0.008	975 ± 102	770 ± 26	2660 ± 24

Table 3. Concentrations and specific radioactivities of InsP₄(s) and InsP₅ in avian erythrocytes incubated *in vitro*

All samples were initially purified on a Partisil 10-SAX anion-exchange h.p.l.c. column and all subsequent steps used a Partisphere 5-WAX anion-exchange h.p.l.c. column. A 0.2 ml portion of packed avian erythrocytes was labelled with [³H]Ins for 6 h (5 mCi/ml) and [³²P]P_i was introduced into the incubation medium (10 mCi/ml final concn.) for the last 50 min of this period. Inositol tetrakis- and pentakisphosphates were extracted and purified from 0.2 ml of packed cells. The ³H/³²P d.p.m. ratios of the purified isomers were measured by liquid scintillation counting. Aliquots of the [³H]Ins[³²P](3,4,5,6)P₄ and [³H]Ins[³²P](1,3,4,5,6)P₅ preparations were partially dephosphorylated to a series of defined intermediates (see Figs. 3 and 5). The remainder of these fractions and all of the [³H]Ins[³²P](1,3,4,6)P₄ and [³H]Ins[³²P](1,3,4,5)P₄ fractions were completely dephosphorylated with alkaline phosphatase and the [³H]Ins recovered was quantified by an enzyme-linked fluorimetric assay. This yielded estimates of the intracellular concentrations of these compounds and of their overall ³H and ³²P specific radioactivities. The data obtained for [³H]Ins[³²P](3,4,5,6)P₄ and [³H]Ins[³²P](1,3,4,5,6)P₅ are also used in Table 8. The majority of the assays were performed in duplicate, with the mean values being shown in the Table. The data for InsP₅ were obtained in triplicate and its concentration and specific radioactivities are presented as means ± S.E.M. (n = 3). The specific radioactivity of the γ-phosphate of ATP was assayed in triplicate in a parallel incubation, and was 617000 d.p.m./nmol (mean of the three determinations). Data from an independent experiment in which the concentration of [³H]Ins was 0.3 times that in the experiment in this Table are also shown (bottom two lines): * specific radioactivities of the inositol phosphates contained in three separate samples of the cell suspension were determined, and are presented as means ± S.E.M.

Inositol phosphate	H.p.l.c. purification steps	³ H/ ³² P ratio	Cellular concn. (nmol/ml)	Specific radioactivity (d.p.m./nmol)	
				³ H	³² P
Ins(1,3,4,6)P ₄	2	9.807	12.0	1112929	231522
Ins(1,3,4,5)P ₄	2	2.766	14.8	2787296	1007699
Ins(3,4,5,6)P ₄	4	13.75	81.9	196925	14322
Ins(1,3,4,5,6)P ₅	2	3.03	1136 ± 41	64267 ± 2889	21210
Ins(3,4,5,6)P ₄	3	—	—	119220 ± 745*	—
Ins(1,3,4,5,6)P ₅	2	—	—	36777 ± 1008*	—

[³H]Ins[³²P](3,4,5,6)P₄ and [³H]Ins[³²P](1,3,4,5,6)P₅. Fig. 2 also defines the time frame within which samples of erythrocytes were subsequently incubated with [³²P]P_i to allow sufficient [³²P]P_i to become incorporated into the inositol phosphates, such that the intramolecular distribution of [³²P]P_i within the inositol phosphates could be determined (see below) while maintaining the maximum possible differences between the specific radioactivities of ³²P precursors and their products (see below).

Cellular concentration and specific radioactivities of [³H]Ins[³²P]P₄s and [³H]Ins[³²P]P₅

[³H]Ins[³²P]P₄s and [³H]Ins[³²P]P₅ which had been extracted and purified from [³H]Ins- and/or [³²P]P_i-labelled avian erythrocytes were quantified both by dual-label liquid scintillation counting and by either phosphate or inositol assay (Tables 2 and 3 respectively). The quantities of the [³H]Ins[³²P]P₄s and [³H]Ins[³²P]P₅ determined by

Table 4. Determination of the specific radioactivities of the individual phosphate groups in [^3H]Ins[^{32}P](1,3,4,5) P_4 derived from avian erythrocytes incubated with [^{32}P] P_i for 90 min

(a) A 0.2 ml portion of packed avian erythrocytes was incubated with [^3H]Ins for 7 h (8 mCi/ml) and with [^{32}P] P_i (10 mCi/ml) for the last 90 min of this period. The suspension was quenched and the [^3H]Ins[^{32}P] P_i s were extracted and purified as described in the Materials and methods section. The [^3H]Ins[^{32}P](1,3,4,5) P_4 peak was isolated (see Figs. 2 and 3), desalted and aliquots were incubated with a rat brain homogenate for 0 or 3 min. The reactions were terminated with HClO_4 and processed for application to a Partisphere 5-SAX anion-exchange h.p.l.c. The column was eluted into 0.4 min fractions and counted for ^3H and ^{32}P radioactivity utilizing standard dual-label liquid scintillation counting techniques as described (results not shown). The dephosphorylation products were designated peaks A to D (from the least retained Ins P_2 through to the residual Ins P_4) and were identified by the following procedure. The peak fractions were pooled and the scintillant and salt were removed. Aliquots from each fraction were oxidized with periodate, reduced and dephosphorylated; the [^3H]polyols recovered (the percentages of the starting ^3H recovered in [^3H]polyols(s) are shown in the Table) were identified on the basis of their retention time on a cation-exchange h.p.l.c. column (in the Pb^{2+} mode) relative to internal [^{14}C]inositol and [^{14}C]glucitol. The lack of a 2-phosphate in the parent Ins P_4 was established by resolution of inositol monophosphates produced after hydrolysis in 10 M- NH_4OH for 60 h at 110 °C on an anion-exchange h.p.l.c. column (results not shown). The structure of the more-retained Ins P_2 (peak B) was deduced to be Ins(3,4) P_2 on the basis of the fact that the starting material contained no 6-phosphate. The stereochemical series to which the [^3H]polyols belonged was determined by the use of L-iditol dehydrogenase. (b) This shows the derivation of the relative and absolute specific radioactivities of the individual monoester phosphates of this sample of [^3H]Ins[^{32}P](1,3,4,5) P_4 from the data in section (a) of this Table. Essentially, the relative specific radioactivities of the individual phosphates (i.e. their $^{32}\text{P}/^3\text{H}$ ratios) are derived from the differences between the $^{32}\text{P}/^3\text{H}$ ratios of defined dephosphorylation products. The absolute specific radioactivities of the individual phosphate moieties (in units of d.p.m./nmol) were calculated from knowledge of the relative specific radioactivities and measurement of the concentrations of Ins(1,3,4,5) P_4 in a parallel suspension of erythrocytes (14.8 μM , see Table 3). The absolute specific radioactivity of ^{32}P in the γ -phosphate of ATP was 279 430 d.p.m./nmol.

(a)	Total ^3H recovered in inositol phosphate (d.p.m.)	Chromatographic identity	$^{32}\text{P}/^3\text{H}$ ratio	Polyol(s) recovered	Recovery of polyols (%)	Identity of inositol phosphate
A	1803 812	Ins P_2	0.0220	Adonitol	97	Ins(1,3) P_2
B	97 494	Ins P_2	0.0306	Threitol	84	Ins(3,4) P_2
C	903 371	Ins P_3	0.0311	L-Altritol	80.5	Ins(1,3,4) P_3
D	573 980	Ins P_4	0.0418	Inositol	78	Ins(1,3,4,5) P_4
Zero time incubation		Ins P_4	0.0435	—	—	—

(b)	Position of phosphate	Derivation of $^{32}\text{P}/^3\text{H}$ for defined phosphate	$^{32}\text{P}/^3\text{H}$ ratio	^{32}P distribution (% of total)	Absolute specific radioactivity of ^{32}P in defined phosphate (d.p.m./nmol)
	1	(1,3,4) — (3,4)	0.0005	1.2	4231
	3	(1,3,4,5) — [(1) + (4) + (5)]	0.0215	51.4	181 230
	4	(1,3,4) — (1,3)	0.0091	21.8	76 864
	5	(1,3,4,5) — (1,3,4)	0.0107	25.6	90 262
	Total (1,3,4,5)	Direct	0.0418	100	352 588
					[^3H (d.p.m./nmol) = 8 109 535]

inositol and phosphate assays were in good agreement and suggested that the packed cell concentrations of [^3H]Ins[^{32}P](1,3,4,6) P_4 , [^3H]Ins[^{32}P](1,3,4,5) P_4 , [^3H]Ins[^{32}P](3,4,5,6) P_4 and [^3H]Ins[^{32}P](1,3,4,5,6) P_5 were 12 μM , 14 μM , 81 μM and 1 mM respectively (Tables 2 and 3).

The specific radioactivities of both ^3H and ^{32}P were determined in all four inositol phosphates extracted from avian erythrocytes incubated for various times with the tracers (Tables 2 and 3). The specific radioactivity of the [^3H]Ins derived from these compounds defines a series: [^3H]Ins[^{32}P](1,3,4,5) P_4 > [^3H]Ins[^{32}P](1,3,4,6) P_4 > [^3H]Ins[^{32}P](3,4,5,6) P_4 > [^3H]Ins[^{32}P](1,3,4,5,6) P_5 . This suggests that none of the other [^3H]Ins[^{32}P] P_i s or [^3H]Ins[^{32}P] P_5 could act as the major precursor of [^3H]Ins[^{32}P](1,3,4,5) P_4 although, theoretically, any of the

[^3H]Ins[^{32}P] P_i s could act as the major precursor of [^3H]Ins[^{32}P] P_5 . The overall specific radioactivity of ^{32}P in the Ins P_4 s followed the same pattern as that seen for ^3H , although the overall specific radioactivity of [^3H]Ins[^{32}P] P_5 was higher than that of [^3H]Ins[^{32}P](3,4,5,6) P_4 . In order to interpret the ^{32}P specific radioactivity data (both from the inositol phosphates and from the γ -phosphate of ATP), it was necessary to determine the distribution of the ^{32}P amongst the individual phosphate groups of these compounds.

Analysis of the distribution of ^{32}P amongst the phosphate moieties of ^3H - and ^{32}P -labelled inositol phosphates

Dephosphorylation of [^3H]Ins[^{32}P](1,3,4,5) P_4 . Radiochemically pure (> 99.5 %) [^3H]Ins[^{32}P](1,3,4,5) P_4 de-

Table 5. Measurement of the relative [^{32}P]P_i content of the individual phosphate moieties of [^3H]Ins[^{32}P](1,3,4,6)P₄

[^3H]Ins[^{32}P](1,3,4,6)P₄ was purified from the same batch of [^3H]Ins- and [^{32}P]P_i-prelabelled avian erythrocytes that yielded the [^3H]Ins[^{32}P](1,3,4,5)P₄ and [^3H]Ins[^{32}P](3,4,5,6)P₄ analysed in Tables 4 and 6. An aliquot of this preparation of [^3H]Ins[^{32}P](1,3,4,6)P₄ was incubated with partially purified phosphatase activities from rat brain cytosol [Ins(1,3,4,6)P₄ phosphomonoesterase activities with native relative molecular masses of 31 and 150 kDa; see Stephens *et al.*, 1989]. Incubation of [^3H]Ins[^{32}P](1,3,4,6)P₄ with the 150 kDa Ins(1,3,4,6)P₄ 6-phosphate phosphomonoesterase activity yielded a single [^3H]Ins[^{32}P]P₃ designated C, the ^3H and ^{32}P contents of which were measured by dual-label liquid scintillation counting. It was then extracted from the scintillant in which it had been counted, and desalted. An aliquot of desalted [^3H]Ins[^{32}P]P₃ (C) was oxidized with periodate, reduced and dephosphorylated; polyols were identified as for Table 4. A second aliquot was dephosphorylated with a rat brain cytosol fraction to yield two [^3H]Ins[^{32}P]P₂s that could be resolved by anion-exchange h.p.l.c., designated [^3H]Ins[^{32}P]P₂ (I) (the less-retained isomer) and [^3H]Ins[^{32}P]P₂ (II). After the ^3H and ^{32}P contents had been measured by dual-label liquid scintillation counting, the [^3H]Ins[^{32}P]P₃s were extracted from the scintillation fluid in which they had been counted, and were desalted and converted into identifiable polyols. Incubation of [^3H]Ins[^{32}P](1,3,4,6)P₄ with the 31 kDa Ins(1,3,4,6)P₄ 4-phosphate and 3-phosphate phosphomonoesterase activities (as described in the Materials and methods section) yielded two [^3H]Ins[^{32}P]P₃s that could be resolved by anion-exchange h.p.l.c. and which were designated [^3H]Ins[^{32}P]P₃ (A) and [^3H]Ins[^{32}P]P₃ (B) (in order of increasing retention time). After determining the ^3H and ^{32}P contents of [^3H]Ins[^{32}P]P₃s (A) and (B) by dual-label liquid scintillation counting, they were extracted from the scintillant in which they were counted, desalted and converted into identifiable polyols. The relative, and absolute, specific radioactivities of the individual monoester phosphates of [^3H]Ins[^{32}P](1,3,4,6)P₄ were calculated from the data presented in sections (a) and (b) (see section c) exactly as described for [^3H]Ins[^{32}P](1,3,4,5)P₄. Several independent internal estimates of each of the values could be made in this analysis. The absolute specific radioactivities were calculated assuming that the packed cell concentration of [^3H]Ins[^{32}P](1,3,4,6)P₄ in the erythrocytes used in this analysis was the same as that determined in a parallel experiment (see Table 3), i.e. 12.0 μM . The ^{32}P specific activity of the γ -phosphate of ATP was 279430 d.p.m./nmol.

Origin of inositol phosphates	Peak designation and chromatographic identity	Total ^3H in peak (d.p.m.)	$^{32}\text{P}/^3\text{H}$ ratio	Polyol(s)	Recovery of polyols (%)	Identity
(a) Dephosphorylation of [^3H]Ins[^{32}P](1,3,4,6)P₄						
[^3H]Ins[^{32}P](1,3,4,6)P ₄ + 31 kDa phosphatase	InsP ₃ (A)	1999775	0.0130	D-Altritol	85	Ins(1,3,6)P ₃
	InsP ₃ (B)	111679	0.0067	D-Iditol	40	Ins(1,4,6)P ₃
[^3H]Ins[^{32}P](1,3,4,6)P ₄ + 150 kDa phosphatase	InsP ₃ (C)	817175	0.0146	L-Altritol	96	Ins(1,3,4)P ₃
Residual [^3H]Ins[^{32}P]P ₄ from above	InsP ₄	892655	0.0174	—	—	Ins(1,3,4,6)P ₄
[^3H]Ins[^{32}P]P ₄ (zero-time incubation)	InsP ₄	602795	0.0173	Inositol	78	Ins(1,3,4,6)P ₄
(b) Dephosphorylation of [^3H]Ins[^{32}P](1,3,4)P₃ derived from [^3H]Ins[^{32}P](1,3,4,6)P₄						
[^3H]Ins[^{32}P]P ₃ (C) from rat brain cytosol	InsP ₂ (I)	77736	0.0102	Adonitol	54	Ins(1,3)P ₂
	InsP ₂ (II)	228744	0.0140	Threitol	76	Ins(3,4)P ₂
Residual [^3H]Ins[^{32}P]P ₃ from above	InsP ₃	103798	0.0148	—	—	—

(c) Relative and absolute specific radioactivities of the phosphate groups in [^3H]Ins[^{32}P](1,3,4,6)P₄

Position of phosphate	Derivation of $^{32}\text{P}/^3\text{H}$ for a defined phosphate	$^{32}\text{P}/^3\text{H}$ ratio	^{32}P distribution (% of total)*	Absolute specific radioactivity of ^{32}P in defined phosphate (d.p.m./nmol)
1	(1,3,4) — (3,4)	0.0006	3.5	2433
3	(1,3) — (1)	0.0096	55.4	38521
	(1,3,4,6) — [(1) + (4) + (6)]	0.0094		
	(1,3,4,6) — (1,4,6)	0.0106		
4	(1,3,4,6) — (1,3,6)	0.0043		
	(1,3,4) — (1,3)	0.0044	25.4	17661
6	(1,3,4,6) — (1,3,4)	0.0027	15.6	10847
	(1,3,6) — (1,3)	0.0028		
Total (1,3,4,6)	Direct	0.0173	100	69533

[^3H (d.p.m./nmol) = 4019007]* This analysis assumes the $^{32}\text{P}/^3\text{H}$ ratios of the individual phosphates were 1-P, 0.0006; 3-P, 0.0096, 4-P, 0.0044; 6-P, 0.0027.

rived from [^{32}P]P_i- and [^3H]Ins-prelabelled avian erythrocytes was incubated for 0 or 3 min with a rat brain homogenate under the conditions described above, and the products were resolved on a Partisphere SAX h.p.l.c.

column. The $^{32}\text{P}/^3\text{H}$ content of the original [^3H]Ins[^{32}P](1,3,4,5)P₄ and of the various [^3H]inositol [^{32}P]phosphate metabolites derived from it are presented in Table 4(a) along with the identities of the [^3H]polyols

derived from them (for details, see the Materials and methods section and the legend to Table 4). The mechanism by which these data were used to calculate the relative and absolute specific radioactivities of the individual phosphates of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5)\text{P}_4$ is shown in Table 4(b), along with the results of assays to determine the specific radioactivity of the γ -phosphate of ATP in a parallel acid extract from the same cells. The distribution of ^{32}P in the avian erythrocyte-derived $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5)\text{P}_4$ was consistent with its synthesis from PtdIns via PtdIns4P, PtdIns(4,5) P_2 and Ins(1,4,5) P_3 . The absolute specific radioactivity of the 3-phosphate was 64 % of that of the γ -phosphate of ATP at the same time, suggesting that the turnover of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5)\text{P}_4$ is rapid compared with that of the other $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_4$ s and $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_5$ present in the same cell extracts (see below).

Dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$. $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$ was prepared from the same acid extract as that from which the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5)\text{P}_4$ and $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ (which generated the data shown above and below), were also purified. The purified $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$ was dephosphorylated with partially purified preparations of rat brain cytosol Ins(1,3,4,6) P_4 6-phosphate phosphomonoesterase, Ins(1,3,4,6) P_4 4-phosphate phosphomonoesterase and Ins(1,3,4,6) P_4 3-phosphate phosphomonoesterase activities. (The second and third of these activities were not resolved, but as they yield chromatographically distinct Ins P_3 s the two assays do not interfere; see the Materials and methods section for assay conditions and Stephens *et al.*, 1989, for details of the preparation and characterization of the phosphomonoesterase activities.) An aliquot of the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4)\text{P}_3$ that was derived from $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$ [produced in the Ins(1,3,4,6) P_4 6-phosphate phosphomonoesterase assay] was further dephosphorylated using rat brain cytosol (see the Materials and methods section for details).

The $^{32}\text{P}/^3\text{H}$ contents of the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$ and of both its direct and indirect dephosphorylation products are presented in Tables 5(a) and 5(b) respectively, with the identities of the $[^3\text{H}]\text{polyols}$ derived from each of the metabolites obtained in this analysis. The $^{32}\text{P}/^3\text{H}$ d.p.m. ratios of the individual phosphates of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$ were calculated from the data in Tables 5(a) and 5(b) (see Table 5c). Several independent estimates of the $^{32}\text{P}/^3\text{H}$ ratios of the 3-, 4- and 6-phosphates were possible (all containing sufficient radioactivity to make the measured d.p.m. ratios statistically reliable); all estimates agreed well. Of the total ^{32}P , 55.4 % was unexpectedly located in the 3-phosphate, 25.4 % was in the 4-phosphate, 15.6 % in the 6-phosphate and 3.5 % in the 1-phosphate. The absolute specific radioactivity of the 3-phosphate was only 13.8 % of that of the γ -phosphate of ATP in a parallel acid extract.

Dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$. $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ was purified from the same acid extract of $[^3\text{H}]\text{Ins}$ - and $[^{32}\text{P}]\text{P}_i$ -prelabelled avian erythrocytes as the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5)\text{P}_4$ and $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$ that were the substrates of the analyses presented above. The $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ (99.96 % radiochemically pure) was dephosphorylated with human erythrocyte ghosts (see Fig. 3). The $^{32}\text{P}/^3\text{H}$ d.p.m. contents of the parent $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ and

Fig. 3. Dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ with human erythrocyte ghosts

$[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ was extracted from the same batch of erythrocytes that yielded the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,6)\text{P}_4$ and $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5)\text{P}_4$ analysed in Tables 4 and 5 (see the Materials and methods section) and purified by anion-exchange h.p.l.c. Aliquots of the $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$ were incubated with human erythrocyte ghosts for 0 or 150 min (the zero time sample contained a single peak of ^3H and ^{32}P radioactivity running as an Ins P_4 ; see Table 6) and the reactions were quenched with HClO_4 . The precipitated protein was pelleted by centrifugation and the supernatant was neutralized, applied to an anion-exchange h.p.l.c. column (Partisphere 5-SAX) and eluted. Fractions were collected every 0.4 min and counted for ^3H and ^{32}P radioactivity. Neighbouring vials containing background levels of radioactivity were grouped together and averaged for the purpose of presenting the results. Fractions containing a common peak of radioactivity were pooled, extracted from the scintillation fluid in which they had been counted and desalted. Aliquots of the desalted inositol phosphates in peaks A–G were oxidized with periodate, reduced and dephosphorylated. The identities of the $[^3\text{H}]\text{polyol(s)}$ produced are reported in Table 6. A portion of the salt- and scintillant-free preparation of peak C $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_2$ was incubated with rat brain cytosol in the presence of EDTA (see Fig. 4). Three further experiments, using independent preparations of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)\text{P}_4$, yielded qualitatively similar results (some of the results from one of these additional experiments are represented in Table 8). The $^{32}\text{P}/^3\text{H}$ ratios of the radioactive peaks are shown in the Figure immediately above their corresponding peaks.

its dephosphorylation products are shown in Table 6 along with the $[^3\text{H}]\text{polyols}$ that were derived from the individual metabolites shown in Fig. 3. An aliquot of the $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}$ was rechromatographed with DL- $[^{14}\text{C}]\text{Ins}1\text{P}$ and $[^{14}\text{C}]\text{Ins}4\text{P}$ (as described above); all of the ^3H was eluted with the DL- $[^{14}\text{C}]\text{InsP}$ and was hence assumed to be $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}$. The major $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_2$ produced during the dephosphorylation of

Table 6. Characterization of the inositol phosphates produced during the dephosphorylation of [³H]Ins[³²P](3,4,5,6)*P*₄ and an analysis of the distribution of ³²P amongst the individual phosphate moieties of [³H]Ins[³²P](3,4,5,6)*P*₄

(a) The mixture of inositol phosphates produced during the dephosphorylation of [³H]Ins[³²P](3,4,5,6)*P*₄ was resolved by anion-exchange h.p.l.c. (see Fig. 3). The peak fractions (designated A–G in fig. 3) were pooled and the scintillant and salt were removed. The inositol phosphates were converted to identifiable polyols and recoveries calculated as in Table 4. (b) [³H]Ins[³²P]*P*₂(C) from the dephosphorylation described in (a) and Fig. 3 was extracted from the scintillation fluid in which it was counted, and desalted and incubated with rat brain cytosol in the presence of EDTA for 0 or 10 min (see Fig. 4). The ³H and ³²P contents of the dephosphorylation products were determined by dual-label liquid scintillation counting. (c) The relative specific radioactivities of the individual phosphates of [³H]Ins[³²P](3,4,5,6)*P*₄ were calculated from the data above, assuming that the concentration of Ins(3,4,5,6)*P*₄ in the erythrocytes from which this sample was extracted was 81.9 μM (i.e. the same as that in a parallel batch of erythrocytes; see Table 3).

Peak designation	Chromatographic identity	³² P/ ³ H ratio	Polyol(s)	Recovery of polyols (%)	Identity of inositol phosphate
(a) Dephosphorylation of Ins(3,4,5,6)<i>P</i>₄					
A	Ins <i>P</i>	0.0031	–	–	Ins3 <i>P</i> *
B	Ins <i>P</i> ₂	0.0038	Iditol and altritol	32	Ins(3,6) <i>P</i> ₂
C	Ins <i>P</i> ₂	0.0043	Threitol	90	Ins(3,4) <i>P</i> ₂
D	Ins <i>P</i> ₂	0.0054	–	0.5	D- and/or L-Ins(4,5) <i>P</i> ₂ †
E	Ins <i>P</i> ₃	0.0103	Xylitol	90	Ins(3,4,5) <i>P</i> ₃
F	Ins <i>P</i> ₃	0.0049	–	1	Ins(4,5,6) <i>P</i> ₃
G	Ins <i>P</i> ₄ remaining	0.0100	L-Iditol	70	Ins(3,4,5,6) <i>P</i> ₄
Zero time incubation	Ins <i>P</i> ₄	0.0102	L-Iditol	68	Ins(3,4,5,6) <i>P</i> ₄
(b) Dephosphorylation of Ins<i>P</i>₂ (C)					
	Ins <i>P</i>	0.0032	–	–	Ins3 <i>P</i>
C	Ins <i>P</i> ₂ remaining	0.0043	–	–	Ins(3,4) <i>P</i> ₂
C	Zero time incubation	0.0042	–	–	Ins(3,4) <i>P</i> ₂
(c) Phosphate identity					
Position of phosphate	Derivation of ³² P/ ³ H ratio for individual phosphates	³² P/ ³ H ratio	³² P (% of total)	Specific radioactivity (d.p.m./nmol)	
3	Direct from (3,4,5,6) dephosphorylation	0.00314	30.8	1599	
4	(3,4)–(3)	0.00113	11.1	576	
5	(3,4,5)–(3,4)	0.00603	59.1	3069	
6	(3,4,5,6)–(3,4,5)	–0.00010	–0.98	–50	
Total (3,4,5,6)	Direct	0.0102	100	5193	

[³H] (d.p.m./nmol) = 509956]

* [³H]Ins[³²P]*P* co-chromatographed precisely with [¹⁴C]Ins1*P* (see the Materials and methods section).

† [³H]Ins[³²P]*P*₂ co-chromatographed precisely with Ins[³²P](4,5)*P*₂ [prepared as described in Stephens *et al.* (1989); results not shown].

[³H]Ins[³²P](3,4,5,6)*P*₄ {i.e. [³H]Ins[³²P](3,4)*P*₂} was further dephosphorylated by a rat brain cytosol fraction in the presence of EDTA (see Fig. 4 and Table 6b). The derivation of the ³²P/³H ratios of the individual phosphate groups of [³H]Ins[³²P](3,4,5,6)*P*₄ is shown in Table 6(c). The 5-phosphate contained 59.1 % of the total ³²P in the [³H]Ins[³²P](3,4,5,6)*P*₄; the 3-phosphate, 30.8 %; the 4-phosphate, 11.1 %; and the 6-phosphate, –0.98 %. The absolute specific radioactivity of the 5-phosphate was only 1.1 % of that of the γ-phosphate of ATP in a parallel sample of erythrocytes. The specific

radioactivity of the [³H]Ins moiety in the [³H]Ins[³²P](3,4,5,6)*P*₄ was also lower than that of either of the other [³H]Ins[³²P]*P*₄s (see above).

Dephosphorylation of [³H]Ins[³²P](1,3,4,5,6)*P*₅. H.p.l.c.-purified [³H]Ins[³²P](1,3,4,5,6)*P*₅ (the final preparation was 99.9 % radiochemically pure and was derived from a different erythrocyte incubation than that used for the analyses shown in Table 7 was dephosphorylated with human erythrocyte ghosts and the products were separated by anion-exchange h.p.l.c. (Fig. 5). The ³²P

Fig. 4. Dephosphorylation of the major $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_2$ derived from the dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$

An aliquot of the salt- and scintillant-free preparation of the major $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_2$ (peak C) produced during the dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ (see Fig. 3 and Table 6 for a summary of this and related data) was incubated with rat brain cytosol in the presence of EDTA for 0 or 10 min (the zero-time sample contained a single peak of ^3H and ^{32}P radioactivity which eluted at the time expected for an $\text{Ins}P_2$; see Table 6). The samples were quenched and processed for application to an anion-exchange h.p.l.c. column (a Partisphere 5-SAX column) as described above. The column was eluted as described; fractions were collected every 0.4 min, mixed with scintillation fluid and counted for ^3H (●) and ^{32}P (○) radioactivity as described. Neighbouring vials containing background levels of radioactivity were grouped together and averaged for the purpose of presenting the results. Qualitatively similar results were obtained in three further experiments with independent preparations of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$. The $^{32}\text{P}/^3\text{H}$ ratios of the various metabolites are shown in the Figure, immediately above their corresponding peaks.

and ^3H contents of the individual resolvable metabolites are summarized in Table 7(a), along with the $[^3\text{H}]\text{polyol(s)}$ they yielded upon periodate oxidation, reduction and dephosphorylation. The third most retained $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_3$, derived from the hydrolysis of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ (peak 'G' in Table 7a, which yielded D- $[^3\text{H}]\text{iditol}$ upon periodate oxidation, reduction and dephosphorylation) was further dephosphorylated with human erythrocyte ghosts (see Fig. 6). The ^3H and ^{32}P contents of the residual $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_3(s)$ and the dephosphorylation products generated during the progress of the assay are reported in Table 7(b) (along with the identities of the $[^3\text{H}]\text{polyols}$ derived from them).

The $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_3$ remaining (which was resistant to further dephosphorylation) after the peak G $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_3(s)$ had been further dephosphorylated still yielded D- $[^3\text{H}]\text{iditol}$ upon periodate oxidation, reduction and dephosphorylation. However, if it was boiled in 1.0 M-HCl for 3 min (conditions leading to migration of phosphate groups between *cis*-related hydroxyls;

Stephens *et al.*, 1989), then $[^3\text{H}]\text{Ins}$ was also recovered (41 % of total recovered ^3H). These observations suggest that the original peak G $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_3(s)$ were a mixture of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4,5)P_3$ and $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4,6)P_3$ (32 % and 68 % of the total respectively) and that the erythrocyte-ghost-resistant $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_3$ was $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4,6)P_3$. The facts that the $^{32}\text{P}/^3\text{H}$ ratio of the residual $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_3$ remained constant between 70 and 120 min, and that it was only marginally greater than that of the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4)P_2$ derived from the same starting material, indicate that the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4,6)P_3$ remaining after 120 min incubation with the erythrocyte ghosts was homogeneous and hence its $^{32}\text{P}/^3\text{H}$ content could be used, in conjunction with that of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4)P_2$, to estimate the specific radioactivity of the 6-phosphate of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ (Table 8a).

The principal $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_2$ formed during the dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ (peak A, Table 7) was partially oxidized with sodium periodate, reduced and dephosphorylated (see Fig. 5 and the Materials and methods section for details); the only $[^3\text{H}]\text{polyols}$ recovered were $[^3\text{H}]\text{altritol}$ and $[^3\text{H}]\text{iditol}$. When a portion of the $[^3\text{H}]\text{iditol}$ was mixed with D- $[^{14}\text{C}]\text{iditol}$ and incubated with L-iditol dehydrogenase, it proved resistant to oxidation (2.9 % of the $[^3\text{H}]\text{iditol}$ was oxidized in an assay in which 3.0 % and 99 % of internal D- $[^{14}\text{C}]\text{iditol}$ and L-iditol respectively were oxidized), suggesting that the original inositol bisphosphate was $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4)P_2$. The remainder of the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4)P_2$, derived from $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_5$, was dephosphorylated with a rat brain cytosol fraction in the presence of 2 mM-DL- $\text{Ins}1P$ (see Fig. 7 and the Materials and methods section for details). The major dephosphorylation products were $[^{32}\text{P}]P_1$ and $[^3\text{H}]\text{Ins}[^{32}\text{P}]4P$. The $^{32}\text{P}/^3\text{H}$ ratios of the starting $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,4)P_2$ and the $[^3\text{H}]\text{Ins}[^{32}\text{P}]4P$ are shown in Table 7(b).

The data presented in Tables 7(a)–7(c) were used to derive the $^{32}\text{P}/^3\text{H}$ d.p.m. ratios of the individual phosphates in the sample of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ analysed above (Table 8). Data from an analysis of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ isolated from the same acid extract as the $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ that was analysed above are also presented in Table 8 (the $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ was purified and dephosphorylated precisely as described in the Materials and methods section, Fig. 3 and Table 6). The specific radioactivity of the $[^3\text{H}]\text{Ins}$ moieties in $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ and $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ were determined as described. These data, combined with a knowledge of the distribution of ^{32}P amongst the phosphate groups of these inositol phosphates, allowed the absolute specific radioactivities of their individual phosphates to be calculated directly (see Table 8). The distribution of ^{32}P amongst the phosphate groups of the $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ analysed in this experiment was very similar to that observed in the experiment described in Table 6. The specific radioactivities of all of the individual phosphates in $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ were higher than those of their counterparts in $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$. The specific radioactivity of both the 5-phosphate of $[^3\text{H}]\text{Ins}[^{32}\text{P}](3,4,5,6)P_4$ and the 1-phosphate of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)P_5$ were 1.5 % of that of the γ -phosphate of ATP, suggesting that both of these inositol polyphosphates are turned over substantially more slowly than either $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5)P_4$ or $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,6)P_4$.

Table 7. Assignment of the structures of the inositol phosphates produced during the dephosphorylation of [³H]Ins[³²P](1,3,4,5,6)P₅

[³H]Ins[³²P](1,3,4,5,6)P₅ was purified from avian erythrocytes that had been labelled for 6 h with [³H]Ins and for the last 50 min of this period with [³²P]P_i (see Fig. 5 and Table 3) and incubated with human erythrocyte ghosts for 0 or 150 min. The products were resolved by anion-exchange h.p.l.c. (Fig. 5) into peaks A–I. Section (a) summarizes the relevant data collected for each peak including the ³²P/³H d.p.m. ratio. The [³H]polyols formed upon periodate oxidation, reduction and dephosphorylation were identified and recoveries calculated as in Table 4. The identities of the inositol phosphates in each peak, based on the structural data presented in the Table or Figures, are recorded. Section (b) of the Table summarizes and extends the data in Fig. 7 describing the dephosphorylation of the major [³H]Ins[³²P]P₂ (peak A) produced during the metabolism of the [³H]Ins[³²P]P₅ described in (a) above. Section (c) summarizes and extends the data in Fig. 6 describing the characterization and dephosphorylation of peak G [³H]Ins[³²P]P₃.

Peak	General chromatographic identity	³² P/ ³ H ratio	Polyols recovered	Recovery of polyols (%)	Structure of inositol phosphate(s)
(a) Dephosphorylation of InsP ₅					
A	InsP ₂	0.159	Altritol and D-Iditol	55	Ins(1,4)P ₂
B	InsP ₂	0.244	Xylitol	92	Ins(1,5)P ₂ *
C	InsP ₂	0.119	Threitol	91	D- or L-Ins(3,4)P ₂ *†
D	InsP ₂	0.123	–	< 0.01	D- or L-Ins(4,5)P ₂ *†
E	InsP ₃	0.215	L-Altritol	81	Ins(1,3,4)P ₃
F	InsP ₃	0.254	Xylitol	74	Ins(1,5,6)P ₃ *
G	InsP ₃	0.185	D-Iditol	69	[32 % Ins(1,4,5)P ₃] [68 % Ins(1,4,6)P ₃]
H	InsP ₄	0.269	D-Iditol	49.5	Ins(1,4,5,6)P ₄
I	InsP ₅	0.330	Inositol	68	Ins(1,3,4,5,6)P ₅
Zero time incubation	InsP ₅	0.330	–	–	–
(b) Dephosphorylation of peak A above					
InsP derived from A	InsP	0.0112	–	–	Ins4P*†
Zero time of incubation A	InsP ₂	0.159	–	–	Ins(1,4)P ₂
(c) Dephosphorylation of peak G above					
InsP ₂ derived from G	InsP ₂	0.159	Altritol and D-Iditol	52	Ins(1,4)P ₂
Remaining InsP ₃	InsP ₃	0.162	D-Iditol	42	Ins(1,4,6)P ₃
Zero time incubation of G	InsP ₃	0.186	–	–	{ Ins(1,4,6)P ₃ Ins(1,4,5)P ₃

* Structure of inositol phosphate deduced from available precursors.

† Co-chromatographed with Ins[³²P](4,5)P₂ (results not shown).

‡ Deduced from lack of co-chromatography with D- or L-Ins1P.

DISCUSSION

Interpretation of radiotracer studies

The experimental strategy described in this paper relies on the fact that the rate of turnover of a defined phosphate in an inositol phosphate will depend on the stage in the synthesis of that inositol phosphate at which it was inserted. Thus in a system at steady-state, a phosphate moiety common to an entire series of inositol phosphate intermediates involved in the synthesis of an inositol polyphosphate will turn over more slowly than a phosphate inserted at the final step in the same series of reactions. By using [³²P]P_i to trace P_i metabolism, it is possible to assess the turnover of phosphate-containing compounds in intact cells. At early times after the addition of [³²P]P_i to a steady-state system, the differences in the specific radioactivities of compounds within a precursor/product series will be at a maximum. If all the

phosphates within an inositol polyphosphate are transferred from the same donor (e.g. the γ-phosphate of ATP), then at early times after the addition of [³²P]P_i tracer the phosphate that is inserted last in the synthesis of the parent compound will become labelled with ³²P more quickly than any of the other phosphate moieties. Hence if the identity of the most rapidly labelled phosphate moiety in an inositol polyphosphate can be established, then this defines the last, or in a branched pathway, the principal, phosphorylation reaction that occurs in the synthesis of that inositol polyphosphate. This paper describes experimental strategies that enable this form of analysis to be applied to Ins(1,3,4,5)P₄, Ins(1,3,4,6)P₄, Ins(3,4,5,6)P₄ and Ins(1,3,4,5,6)P₅ labelled by the incubation of avian erythrocytes with [³²P]P_i.

There are several features of these experiments that require consideration if the results they yield are to be accurately interpreted. It is vital that the phosphates

Fig. 5. Dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)\text{P}_5$ by human erythrocyte ghosts

$[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)\text{P}_5$ was extracted from avian erythrocytes which had been prelabelled with $[^3\text{H}]\text{Ins}$ (5 mCi/ml) for 6 h and with $[^{32}\text{P}]\text{P}_i$ (10 mCi/ml) for the last 50 min of this period, and purified with two anion-exchange h.p.l.c. columns (see the Materials and methods section). Aliquots of the radiolabelled InsP_5 were incubated with human erythrocyte ghosts for 0 min (not shown; a single peak of radioactivity eluting at the time expected for an InsP_5 was detected) or 150 min. The reactions were quenched with ice-cold HClO_4 and the samples were processed for application to an anion-exchange h.p.l.c. column (Partisphere SAX), eluted with a phosphate gradient and collected into 0.4 min fractions, which were counted individually for ^3H (●) and ^{32}P (○) radioactivity as described. The vials containing individual peaks of radioactivity were pooled, and the inositol phosphates contained therein were extracted from the scintillation fluid (see the Materials and methods section) and desalted before further analysis. The data shown are from a single experiment; three further experiments yielded similar results. Neighbouring vials containing background levels of radioactivity were grouped together and averaged for the purposes of presenting the results. The total $^{32}\text{P}/^3\text{H}$ ratios of the various metabolites are shown in the Figure, immediately above their corresponding peaks.

under consideration are originally derived from the same metabolic pool of a single species of phosphate donor. All inositol, inositol lipid and inositol phosphate kinases described to date [Ins kinase, English *et al.*, 1966; 'phosphoinositol kinase', Chakrabarti & Biswas, 1981; $\text{Ins}(1,4,5)\text{P}_3$ 3-hydroxykinase, Irvine *et al.*, 1986; $\text{Ins}(1,3,4)\text{P}_3$ 6-hydroxykinase, Shears *et al.*, 1987; $\text{Ins}(3,4,5,6)\text{P}_4$ 1-hydroxykinase, Stephens *et al.*, 1988b; $\text{Ins}(1,3,4,6)\text{P}_4$ 5-hydroxykinase, Stephens *et al.*, 1988c; PtdIns 3-hydroxykinase, Whitman *et al.*, 1988; PtdIns 4-hydroxykinase and $\text{PtdIns}4\text{P}$ 5-hydroxykinase, Hokin & Hokin, 1964; diacylglycerol kinase, glycerol kinase and phosphofructokinase, Strickland, 1962] are ATP-dependent and, furthermore, the addition of a range of other potential phosphate donors (phosphoenol pyruvate, 2,3-diphosphoglycerate, creatine phosphate, CTP and GTP)

Fig. 6. Metabolism of the $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_3$ s produced during the dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)\text{P}_5$ by human erythrocyte ghosts

An aliquot of the $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_3$ s designated peak G ($^{32}\text{P}/^3\text{H}$ ratio of 0.185; see Fig. 5 and Table 7), produced during the dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5,6)\text{P}_5$ with human erythrocyte ghosts, was extracted from the scintillation fluid in which it had been counted, desalted and incubated with human erythrocyte ghosts for 0 min (a), 75 min (results not shown) or 120 min (b). The products of the dephosphorylation were resolved on an anion-exchange h.p.l.c. column and counted for ^3H (●) and ^{32}P (○) radioactivity as described. Qualitatively similar results were obtained in one further experiment utilizing $[^3\text{H}]\text{Ins}[^{32}\text{P}]\text{P}_3$ s derived from an independent preparation of chick erythrocytes. Neighbouring vials containing background levels of radioactivity were pooled together and averaged for the purpose of presentation. The $^{32}\text{P}/^3\text{H}$ d.p.m. ratios of the individual metabolites are shown in the Figure immediately above their corresponding peak. See Table 7 for a summary of these and related data.

to avian erythrocyte lysates with a mixture of $[^3\text{H}]\text{InsP}(s)$ (derived from $[^3\text{H}]\text{Ins}$ -prelabelled avian erythrocytes) failed to fuel any phosphorylation reactions that did not occur in the presence of ATP alone (L. Stephens, unpublished work). On the possibility that there may exist different metabolic pools of the phosphate donor through which $[^{32}\text{P}]\text{P}_i$ enters the inositol phosphate (leading to an ambiguity in the interpretation of the results), it should be pointed out that mature avian erythrocytes are not known to store or secrete adenine nucleotides. Furthermore, the only intracellular organelles they contain are a functionally dormant, highly condensed nucleus and a very small number of mitochondria and undefined membranous organelles (Beam *et al.*, 1979). Finally, the metabolism of avian erythrocytes is most closely related to that of mammalian erythrocytes, which have been

Fig. 7. Further metabolism of the major $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_2$ produced during the dephosphorylation of $[^3\text{H}]\text{Ins}[^{32}\text{P}]P_3$ by human erythrocyte ghosts

Portions of the scintillant- and salt-free inositol bisphosphate fraction (peak A, Fig. 5 and Table 7) were incubated with rat brain cytosol in the presence of 2 mM-DL-Ins1P for 0 (results not shown) or 4 min. The reactions were quenched and processed for application to an anion-exchange h.p.l.c. column as described. The column was eluted with a phosphate gradient (results not shown; a single peak of ^3H and ^{32}P radioactivity eluted from the column at the time expected for an $\text{Ins}P_2$), or, after mixing the sample with $[^{14}\text{C}]\text{Ins}3P$, with an acetate gradient as described in the Materials and methods section [subsequent washing of the column with 0.5 M-(NH_4) $_2\text{HPO}_4$, pH 3.8, containing H_3PO_4 , 25 °C, eluted no further radioactivity; 96 % of the ^3H radioactivity applied to the column was recovered]. The column eluate was collected into 0.4 min fractions which were individually counted for ^{32}P (●), ^{14}C (Δ) and ^3H (○) radioactivity utilizing standard liquid scintillation counting techniques. Neighbouring vials, containing background levels of radioactivity, were pooled together and averaged for the purpose of presentation. The total $^{32}\text{P}/^3\text{H}$ d.p.m. ratios of the various metabolites are shown in the Figure immediately above their corresponding peaks.

shown to metabolize ATP, within a time scale of 2–3 min, in a homogeneous manner (Beutler *et al.*, 1978). Taken together, these observations suggest that, in avian erythrocytes, ATP is likely to be the direct precursor of all the phosphate moieties in the inositol polyphosphates (with the exception of those inositol phosphates which possess a 1-phosphate derived from PtdIns ; in these cases, ATP is only an indirect precursor; see below) and, moreover, that ATP itself is probably homogeneously metabolized. Although a kinetic analysis of experiments of this design is simplified enormously if steady state conditions can be shown to prevail, if the $[^{32}\text{P}]\text{P}_i$ content of an inositol phosphate is considered in isolation, then it would still be expected to be determined by the factors considered above, regardless of the existence of steady-state conditions. However, if steady-state conditions do not prevail, the differences in specific radioactivity upon which the analyses depend may be less readily detected, and the ^{32}P specific radioactivities of different inositol phosphates cannot be compared directly. Nevertheless, assays for the level of ATP and the inositol polyphosphates suggest that ATP and inositol polyphosphate metabolism in avian erythrocytes is in a stable state (see Fig. 1 and Tables 2 and 3; further unreported experiments

yielded values for the concentrations of the inositol phosphates in different cell preparations and at different times within the course of these experiments which varied by a maximum of 20 % from those shown).

The experiments reported initially generate estimates of the relative ^{32}P content of the individual phosphate moieties of a defined inositol phosphate. Although this pattern indicates the sequence in which those phosphates were incorporated, it cannot be compared with any other inositol polyphosphate other than by matching the rank order of relative specific radioactivities of the phosphate moieties common to the pair of compounds under consideration. Clearly, if the rank order of the relative specific radioactivities of phosphate groups common to two inositol phosphates do not correlate, then this suggests that they are not directly related. Conversely, though, if their rank orders do correlate, this does not define the nature of the metabolic relationship between the two inositol phosphates, as a coincidence in rank order of common phosphates could emerge from either a dephosphorylation or a phosphorylation reaction. To solve this problem and to allow a 'direction of flow' to be established, the absolute specific radioactivities of the $[^3\text{H}]\text{Ins}$, and/or individual monoester $[^{32}\text{P}]\text{phosphate}$ moieties must be determined.

Finally, and fairly obviously, these experiments only directly describe the histories of phosphates still present in the inositol phosphate being analysed; they cannot directly report on phosphates inserted and subsequently removed (although if other phosphates are 'carried through' this dephosphorylation it may be possible to gain information about the intermediates involved) or on the addition of non-phosphate-containing moieties (although the turnover of the intermediates with which the non-phosphate-containing moiety is associated could be analysed).

In all of the experiments reported, the erythrocytes were incubated in media which raised the initial rate of $[^{32}\text{P}]\text{P}_i$ entry into the cells by approx. 9-fold, without adversely affecting their ability to maintain ATP levels in the normal range. This enhanced rate of $[^{32}\text{P}]\text{P}_i$ turnover across the plasma membrane allowed the time in which the cells were incubated with $[^{32}\text{P}]\text{P}_i$ tracer to be substantially reduced, hence increasing the relative differences in specific radioactivity between precursors and products that the experiments aimed to quantify. This effect can only be exploited in cells which utilize an anion transporter to catalyse P_i turnover at their plasma membrane. Avian erythrocytes are well endowed with an anion transporter and this fact, combined with other advantages (they contain large quantities of $\text{Ins}P_5$, but only trace amounts of $\text{Ins}P_6$; they possess a 'simple' structure and metabolism, but express a receptor-linked phosphoinositidase C activity; they are available in large quantities and yield an easily made, resilient, homogeneous cell preparation that is particularly appropriate for incubation *in vitro*; and they readily incorporate $[^3\text{H}]\text{Ins}$ into inositol phospholipids and phosphates) led to their selection for a detailed study of inositol phosphate metabolism.

Product-precursor relationships amongst the inositol polyphosphate species analysed

$\text{Ins}(1,3,4,5)P_4$. Analysis of $[^3\text{H}]\text{Ins}[^{32}\text{P}](1,3,4,5)P_4$ extracted from avian erythrocytes (prelabelled with radioactive tracers as described above) showed that it has the

Table 8. Specific radioactivities of the individual phosphate groups of [^3H]Ins[^{32}P](1,3,4,5,6) P_5 and [^3H]Ins[^{32}P](3,4,5,6) P_4 derived from the same sample of erythrocytes

(a) Using knowledge of the structure of some of the dephosphorylation products resulting from the hydrolysis of [^3H]Ins[^{32}P] P_5 by human erythrocyte ghosts (see Fig. 5 and Table 7) and the $^{32}\text{P}/^3\text{H}$ d.p.m. ratio of these structurally defined [^3H]inositol [^{32}P]phosphates, it is possible to calculate the relative [^{32}P] P_i content of each of the individual phosphates in the parent inositol phosphate (Table 4). The absolute specific radioactivities of the parent [^3H]Ins[^{32}P](3,4,5,6) P_4 and [^3H]Ins[^{32}P](1,3,4,5,6) P_5 were measured by dephosphorylating them and assaying an aliquot of the hydrolysates for *myo*-inositol (as described in the Materials and methods section). The specific radioactivity of the [^3H]Ins in the starting inositol phosphates could be calculated directly; that of [^{32}P] P_i in the entire molecule and in the individual monoester phosphate moieties could be calculated via knowledge of the $^3\text{H}/^{32}\text{P}$ d.p.m. ratio of the purified parent inositol phosphate. (b) [^3H]Ins[^{32}P](3,4,5,6) P_4 purified from the same extract as the [^3H]Ins[^{32}P] P_5 used in the analysis described in (a) and Table 7 was dephosphorylated precisely as defined for the sample of [^3H]Ins[^{32}P](3,4,5,6) P_4 , the analysis of which is described in Fig. 3 and Table 6. The relative and absolute specific radioactivities of the parent Ins P_4 and of its individual monoester phosphate groups were measured using a strategy identical to that described in (a). The ^{32}P specific radioactivity of the γ -phosphate of ATP was 617211 d.p.m./nmol.

Location of phosphate moiety	Derivation of $^{32}\text{P}/^3\text{H}$ ratio for individual phosphates	$^{32}\text{P}/^3\text{H}$ ratio	^{32}P (% of total)	Specific radioactivity (d.p.m./nmol)	
				^{32}P	^3H
(a) [^3H]Ins[^{32}P](1,3,4,5,6) P_5					
1	(1,4)–(4)	0.148	44.8	9502	–
3	(1,3,4,5,6)–(1,4,5,6)	0.061	18.5	3924	–
4	Direct	0.011	3.4	721	–
5	(1,4,5,6)–(1,4,6)	0.107	32.4	6872	–
6	(1,4,6)–(1,4)	0.003	0.91	193	–
Total (1,3,4,5,6)	Direct	0.330	100	21210	64267
(b) [^3H]Ins[^{32}P](3,4,5,6) P_4					
3	Direct	0.021	28.8	4298	–
4	(3,4)–(3)	0.0096	13.2	1970	–
5	(3,4,5)–(3,4)	0.044	60.8	9073	–
6	(3,4,5,6)–(3,4,5)	–0.002	–2.7	–402	–
Total (3,4,5,6)	Direct	0.0730	100	14922	196925

expected structure (see the Introduction section). The assignment hinges on the demonstration that the [^3H]Ins[^{32}P](1,3,4) P_3 produced upon its dephosphorylation yielded L-[^3H]altritol; hence both the parent inositol tris- and tetrakisphosphate must have a D-4 phosphate. The distribution of ^{32}P amongst the phosphate moieties of the [^3H]Ins[^{32}P](1,3,4,5) P_4 was consistent with its synthesis via PtdIns, PtdIns4P, PtdIns(4,5) P_2 and Ins(1,4,5) P_3 [although, as was pointed out above, the presence of a diacylglycerol moiety in the early stages of the synthesis of Ins(1,3,4,5) P_4 , and the independent existence of PtdIns(4,5) P_2 and Ins(1,4,5) P_3 , go 'unnoticed' in this analysis]. These observations, along with evidence that [^3H]Ins(1,4,5) P_3 is present in [^3H]Ins-prelabelled avian erythrocytes (Stephens *et al.*, 1989), confirm the expectation that cellular Ins(1,3,4,5) P_4 is derived from Ins(1,4,5) P_3 (Irvine *et al.*, 1986). The much lower specific radioactivities of the [^3H]Ins and individual phosphate moieties, as well as the precise distribution of the [^{32}P] P_i within [^3H]Ins[^{32}P](1,3,4,6) P_4 , [^3H]Ins[^{32}P](3,4,5,6) P_4 and [^3H]Ins[^{32}P](1,3,4,5,6) P_5 , completely rule out any unexpected isomerization or dephosphorylation reactions as sources of cellular Ins(1,3,4,5) P_4 .

Although the data from individual experiments only describe the ^{32}P content of [^3H]Ins[^{32}P](1,3,4,5) P_4 at a single time, several features in the data are worthy of note. First, the specific radioactivities of the 4- and 5-phosphates are particularly close, compared with the

relative specific radioactivities of other 'nearest-neighbour' phosphate pairs in [^3H]Ins[^{32}P](1,3,4,5) P_4 or in any of the other inositol polyphosphates investigated, and this would be consistent with the rapid turnover of the pool of PtdIns4P that acts as a precursor for Ins(1,3,4,5) P_4 . [If the system is at steady-state and no 'substrate' or 'futile' cycle exists between PtdIns4P and PtdIns(4,5) P_2 , this would mean that the relevant pool of PtdIns4P must be very small.]

Secondly, the very low, but significant, ^{32}P content of the 1-phosphate would be consistent with a report (Harden *et al.*, 1987) that avian erythrocytes, unlike human erythrocytes, turn over the phosphodiester moiety of PtdIns. The fact that the specific radioactivity of the 1-phosphate is relatively low is not necessarily a consequence of the large pool of PtdIns in avian erythrocytes because the phosphodiester phosphate of PtdIns is derived from the phosphate moiety of phosphatidic acid (and hence possibly from glycerol 3-phosphate), and it is the combination of the turnovers of these phosphates that determine the way in which the 1-phosphate of Ins(1,3,4,5) P_4 becomes labelled.

Ins(1,3,4,6) P_4 . Ins(1,3,4,6) P_4 was originally described as the product of the ATP-dependent phosphorylation of Ins(1,3,4) P_3 (Shears *et al.*, 1987). An activity which phosphorylates Ins(1,3,4) P_3 to yield Ins(1,3,4,6) P_4 has also been identified in adrenal glomerulosa cells and rat brain homogenates (Balla *et al.*, 1987; Stephens *et al.*,

1988c). Avian erythrocytes also possess a soluble ATP-dependent $\text{Ins}(1,3,4)\text{P}_3$ 6-hydroxykinase activity which can be completely resolved from $\text{Ins}(3,4,5,6)\text{P}_4$ 1-hydroxykinase, $\text{Ins}(1,3,4,6)\text{P}_4$ 5-hydroxykinase and $\text{Ins}(1,4,5)\text{P}_3$ 3-hydroxykinase activities (L. Stephens, unpublished work). As $[\text{^3H}]\text{Ins}$ -prelabelled avian erythrocytes contain $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ (Stephens *et al.*, 1989), cellular $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ was anticipated to be derived from the action of an $\text{Ins}(1,3,4)\text{P}_3$ 6-hydroxykinase activity on $\text{Ins}(1,3,4)\text{P}_3$. The results outlined above suggest that this is not the case. The fact that the 3-phosphate of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ contains 55% of the total ^{32}P in this InsP_4 suggests that the major precursor of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ in intact avian erythrocytes is $[\text{^3H}]\text{Ins}[\text{^32P}](1,4,6)\text{P}_3$. Relatively low levels of $[\text{^3H}]\text{Ins}(1,4,6)\text{P}_3$ have been detected in $[\text{^3H}]\text{Ins}$ -prelabelled avian erythrocytes (Stephens *et al.*, 1989), adding to the credibility of this unexpected result. The absolute specific radioactivity of the individual phosphate groups and the inositol moieties of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5)\text{P}_4$, $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$, and $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5,6)\text{P}_5$, derived from the same acid extracts as the $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ above, are such that dephosphorylation or isomerization of these compounds could not account for the labelling of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ {the only potential exception being a series of isomerizations of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5)\text{P}_4$ }.

If the data describing the ^{32}P labelling of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ are simply extrapolated back to free inositol, they suggest a sequential phosphorylation of the 1, 6 and 4 hydroxyl moieties. In principle, this pattern could be generated via a series of inositol phospholipid kinases and a phospholipase C activity. However, since no $\text{Ptd}[\text{^3H}]\text{Ins}(4,6)\text{P}_2$ or $\text{Ptd}[\text{^3H}]\text{InsP}_3$ could be detected in $[\text{^3H}]\text{Ins}$ -prelabelled avian erythrocytes (Stephens *et al.*, 1989; L. Stephens, unpublished work), this alternative explanation for the ^{32}P labelling data described above is unlikely to be correct.

$[\text{^3H}]\text{Ins}(1,4,6)\text{P}_3$ is a minor product of the dephosphorylation of $[\text{^3H}]\text{Ins}(1,3,4,6)\text{P}_4$ in rat brain cytosol and avian erythrocyte lysates (Stephens *et al.*, 1989). Although the first-order rate constant for this reaction is very low in these cell-free systems, the possibility that this reaction has a significant flux *in vivo* and forms part of a substrate cycle cannot be assessed until the turnover of the inositol and phosphate moieties of cellular $\text{Ins}(1,4,6)\text{P}_3$ have been measured.

$\text{Ins}(3,4,5,6)\text{P}_4$. Whereas $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4,6)\text{P}_4$ can be readily synthesized in cell-free systems, from either cell-derived $[\text{^3H}]\text{InsP}_3$ s or pure $[\text{^3H}]\text{InsP}_3$ isomers prepared *in vitro*, efforts to synthesize significant quantities of $[\text{^3H}]\text{Ins}(3,4,5,6)\text{P}_4$ by these mechanisms have failed. In avian erythrocytes briefly labelled with $[\text{^32P}]\text{P}_i$, both the total radioactivity accumulated in, and the specific radioactivity of, $\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ are substantially lower than those of either $\text{Ins}[\text{^32P}](1,3,4,5)\text{P}_4$ or $\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$, so it may be that the former isomer's relatively low rate of metabolism caused the experiments designed to detect its cell-free synthesis to fail. Analysis of the incorporation of radioactive tracers into $\text{Ins}(3,4,5,6)\text{P}_4$ in intact cells is one of the few remaining experimental strategies with which to probe its synthesis.

In four independent preparations of avian-erythrocyte-

derived $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ (the data from two of these are presented in the Results section), the rank order of the phosphates in terms of their specific radioactivities was $5 > 3 > 4 > 6$. The specific radioactivity of the $[\text{^3H}]\text{Ins}$ moiety in the avian erythrocyte $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ preparations was significantly lower than that in either of the other $[\text{^3H}]\text{Ins}[\text{^32P}]\text{P}_4$ s. These results make it possible to state that $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ could not be derived from either $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5)\text{P}_4$ or $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ by a single isomerization, nor could it be generated by a single dephosphorylation-phosphorylation loop from $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$, although this latter mechanism could theoretically generate $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ with an appropriate 'tracer profile' from $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ (see below). The pattern of labelling observed in $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ could be generated by dephosphorylation of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5,6)\text{P}_5$ (by removal of the 1-phosphate, see below). The specific radioactivities of both the $[\text{^3H}]\text{Ins}$ moiety and all of the phosphate groups common to $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ and $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5,6)\text{P}_5$ are higher in the former metabolite, suggesting that, at most, only a small proportion of this $[\text{^3H}]\text{Ins}[\text{^32P}]\text{P}_4$ is derived from the dephosphorylation of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5,6)\text{P}_5$. Thus the most likely origin of cellular $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ is phosphorylation of the 5-hydroxyl group of $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,6)\text{P}_3$. $[\text{^3H}]\text{Ins}(3,4,6)\text{P}_3$ comprises approx. 5–10% of total $[\text{^3H}]\text{InsP}_3$ s extracted from $[\text{^3H}]\text{Ins}$ -prelabelled avian erythrocytes (Stephens *et al.*, 1989). A set of potential cellular sources of $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,6)\text{P}_3$ could be defined that would be analogous to that outlined for $[\text{^3H}]\text{Ins}[\text{^32P}](1,4,6)\text{P}_3$ above, although unlike the latter, $[\text{^3H}]\text{Ins}(3,4,6)\text{P}_3$ is one of the major dephosphorylation products of $[\text{^3H}]\text{Ins}(1,3,4,6)\text{P}_4$ in avian erythrocyte lysates (Stephens *et al.*, 1989). Furthermore, the specific radioactivity of the $[\text{^3H}]\text{Ins}$ moiety and of all of the relevant phosphate groups in $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ and $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ would be consistent with $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ being derived by a dephosphorylation-phosphorylation loop from $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$.

$\text{Ins}(1,3,4,5,6)\text{P}_5$. The specific radioactivity of the inositol moiety in $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5,6)\text{P}_5$ was lower than that of any of the other inositol phosphates studied, any of which could, therefore, act as precursors of the $[\text{^3H}]\text{Ins}[\text{^32P}]\text{P}_5$. The pattern of labelling of the individual phosphate groups of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5,6)\text{P}_5$ suggests that its major precursor is $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ and, therefore, that the flux through $\text{Ins}(3,4,5,6)\text{P}_4$ 1-hydroxykinase is substantially greater than through $\text{Ins}(1,3,4,6)\text{P}_3$ 5-hydroxykinase. Although the specific radioactivity of the 5-phosphate in $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ is higher than that in $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5,6)\text{P}_5$ {and so are all of the other pairs of homologous phosphates; an outcome which would be expected if $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$ is the precursor of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,5,6)\text{P}_5$ }, the ratio of the specific radioactivities of the 5-phosphates in these two polyphosphates is smaller than that of their $[\text{^3H}]\text{Ins}$ specific radioactivities. This could be a consequence of a contribution to the specific radioactivity of the 5-phosphate (and to all of the other phosphates common to both polyphosphates) made by the phosphorylation of the 5-hydroxyl group of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$. As the $^{32}\text{P}/^3\text{H}$ ratio of $[\text{^3H}]\text{Ins}[\text{^32P}](1,3,4,6)\text{P}_4$ was significantly higher than that of $[\text{^3H}]\text{Ins}[\text{^32P}](3,4,5,6)\text{P}_4$, this would

tend to reduce the difference in the specific radioactivities of the phosphate moieties between [^3H]Ins[^{32}P](3,4,5,6) P_4 and [^3H]Ins[^{32}P](1,3,4,5,6) P_5 by more than the difference in their [^3H]Ins specific radioactivities. Thus this 'anomalous' differential in the [^3H]Ins and [^{32}P] P_i specific radioactivities of [^3H]Ins[^{32}P](3,4,5,6) P_4 and [^3H]Ins[^{32}P](1,3,4,5,6) P_5 may be evidence that a relatively small proportion of [^3H]Ins[^{32}P](1,3,4,5,6) P_5 is synthesized directly from [^3H]Ins[^{32}P](1,3,4,6) P_4 .

Concluding remarks

The existence of organized enzyme complexes catalysing the multistep synthesis of various metabolites has been described (for a review of this literature, see Srere, 1987). The holoenzymes responsible are often composed of weakly associated proteins which are commonly considered to be independent activities on the basis of, for example, pharmacological and chromatographic data that resolve or distinguish enzyme activities capable of catalysing the individual steps. Characteristic features of such pathways include relatively low levels of a series of intermediates and apparently 'inefficient' use of exogenously supplied intermediates, compared with the pathway's overall starting material or in the intact cell. Whether such a multifunctional complex catalyses some of the reactions considered here [for example the insertion of the 6-4-3 sequence of phosphates into Ins(1,3,4,6) P_4 and Ins(3,4,5,6) P_5] is not yet clear. Ins(1,3,4,5,6) P_5 and Ins P_6 are now known to be relatively ubiquitous cellular components (Szwergold *et al.*, 1987), yet little is known of their cellular functions or of how they are made. Ins P_6 is a major phosphoric ester in plant seeds, where it has been suggested to function as a phosphorus reserve, and Ins(1,3,4,5,6) P_5 occurs at very high concentrations in avian erythrocytes and may function as an allosteric regulator of oxygen binding to haemoglobin as does 2,3-bisphosphoglycerate in mammalian erythrocytes (Cosgrove, 1980). More recently, Vallejo *et al.* (1987) have proposed extracellular roles for Ins(1,3,4,5,6) P_5 and Ins P_6 which exert potent excitatory effects when injected into a brainstem nucleus involved in cardiovascular and respiratory control. However, the widespread distribution of inositol polyphosphates suggests a more general function in addition to the specific proposals noted above.

Results reported here define probable precursors in the synthesis of three cellular inositol tetrakisphosphates and suggest that Ins(3,4,5,6) P_4 is the major precursor of Ins(1,3,4,5,6) P_5 in these cells. As Ins(3,4,5,6) P_4 is the major Ins P_4 in unstimulated mouse macrophages (Stephens *et al.*, 1988a), and since an Ins(3,4,5,6) P_4 1-hydroxykinase is widely distributed in mammalian tissues (Stephens *et al.*, 1988b) the pathways of Ins(1,3,4,5,6) P_5 synthesis in mammalian and avian cells may be similar if not identical.

REFERENCES

- Baginski, E. S., Zak, B. & Foq, P. P. (1967) *Clin. Chem.* **13**, 326-330
- Balla, T., Gullemette, G., Baukal, A. J. & Catt, K. J. (1987) *J. Biol. Chem.* **262**, 9952-9955
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466-468
- Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211-215
- Beam, K. G., Seth, L. A., Palade, G. E. & Greengard, P. (1979) *J. Cell Biol.* **83**, 1-15
- Beutler, E., Guinto, E., Kuhl, W. & Matsumoto, F. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2825-2828
- Chakrabarti, S. & Biswas, B. B. (1981) *Ind. J. Biochem. Biophys.* **18**, 398-401
- Cosgrove, D. J. (1980) in *Inositol Phosphates* (Cosgrove, D. J., ed.), Elsevier, Amsterdam
- English, P. D., Deitz, M. & Albersheim, P. (1966) *Science* **151**, 198-199
- Galliard, J., Michell, R. H. & Hawthorne, J. N. (1965) *Biochim. Biophys. Acta* **106**, 551-563
- Harden, T. K., Stephens, L. R., Hawkins, P. T. & Downes, C. P. (1987) *J. Biol. Chem.* **262**, 9057-9061
- Hawkins, P. T., Kirk, C. J. & Michell, R. H. (1984) *Biochem. J.* **218**, 785-793
- Hokin, L. E. & Hokin, M. R. (1964) *Biochem. Biophys. Acta* **84**, 563-575
- Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631-634
- King, C. E., Stephens, L. R., Hawkins, P. T., Guy, G. & Michell, R. H. (1987) *Biochem. J.* **244**, 209-217
- MacGregor, L. C. & Matschinsky, F. M. (1984) *Anal. Biochem.* **141**, 382-389
- Sharpe, E. S. & McCarl, R. L. (1982) *Anal. Biochem.* **124**, 421-424
- Shears, S. B., Parry, J. B., Tang, E. K. Y., Irvine, R. F., Michell, R. H. & Kirk, C. J. (1987) *Biochem. J.* **246**, 139-147
- Srere, P. A. (1987) *Annu. Rev. Biochem.* **56**, 89-124
- Stephens, L. R., Hawkins, P. T., Carter, A. N., Chahwala, S. B., Morris, A. J., Whetton, A. D. & Downes, C. P. (1988a) *Biochem. J.* **249**, 271-282
- Stephens, L. R., Hawkins, P. T., Morris, A. J. & Downes, C. P. (1988b) *Biochem. J.* **249**, 283-292
- Stephens, L. R., Hawkins, P. T., Barker, C. J. & Downes, C. P. (1988c) *Biochem. J.* **253**, 721-733
- Stephens, L. R., Hawkins, P. T. & Downes, C. P. (1989) *Biochem. J.* **259**, 267-276
- Strickland, K. P. (1962) *Can. J. Biochem. Physiol.* **40**, 247-254
- Szwergold, B. S., Graham, R. A. & Brown, T. R. (1987) *Biochem. Biophys. Res. Commun.* **149**, 874-881
- Vallejo, M., Jackson, T., Lightman, S. & Hanley, M. R. (1987) *Nature (London)* **330**, 656-658
- Whitman, M., Downes, C. P., Keeler, M., Keller, T. & Cantley, L. (1988) *Nature (London)* **332**, 644-646
- Whittam, R. (1964) *Transport and Diffusion in Red Blood Cells*, pp. 28-29, Edward Arnold, London