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# An analysis of *myo*-[$^3$H]inositol trisphosphates found in *myo*-[$^3$H]inositol prelabelled avian erythrocytes

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Evidence is presented to show that acid extracts of avian erythrocytes prelabelled for 24–48 h with *myo*-[$^3$H]inositol contain the following *myo*-[$^3$H]inositol trisphosphates (expressed as a percentage of total *myo*-[$^3$H]inositol trisphosphates extracted): 36 % *myo*-[$^3$H]inositol 1,4,5-trisphosphate; 33.7 % *myo*-[$^3$H]inositol 1,3,4-trisphosphate; 13 % *myo*-[$^3$H]inositol 3,4,5-trisphosphate; 9.7 % *myo*-[$^3$H]inositol 3,4,6-trisphosphate; 4.4 % *myo*-[$^3$H]inositol 1,4,6-trisphosphate and 3.3 % *myo*-[$^3$H]inositol 1,3,6-trisphosphate. The only phosphatidyl-*myo*-[$^3$H]inositol bisphosphate that could be detected in [$^3$H]Ins-prelabelled avian erythrocytes was phosphatidyl-*myo*-[$^3$H]inositol 4,5-bisphosphate. Cellular *myo*-[$^3$H]inositol 3,4,5-trisphosphate may be synthesized by dephosphorylation of *myo*-[$^3$H]inositol 3,4,5,6-tetrakisphosphate. D- and L-*myo*-[$^3$H]inositol 1,4,6-trisphosphate and D- and L-*myo*-[$^3$H]inositol 1,3,6-trisphosphate may be dephosphorylation products of *myo*-[$^3$H]inositol 1,3,4,6-tetrakisphosphate.

## INTRODUCTION

Three isomers of Ins$P_3$ have been tentatively identified in aqueous extracts of animal cells: Ins(1:2-cyclic,4,5)$P_3$, Ins(1,4,5)$P_3$ and Ins(1,3,4)$P_3$ (Irvine *et al.*, 1984; Dixon & Hokin, 1987; Wong *et al.*, 1988). Ins(1,4,5)$P_3$ and Ins(1:2-cyclic,4,5)$P_3$ are products of an agonist-sensitive phosphoinositidase C(s), Ins(1,3,4)$P_3$ being indirectly derived from Ins(1,4,5)$P_3$ by sequential phosphorylation and dephosphorylation of the 3 and 5 substitution sites respectively (Batty *et al.*, 1985; Irvine *et al.*, 1986). With the recent description of Ins$P_6$, Ins$P_5$, Ins(1,3,4,6)$P_4$ and Ins(3,4,5,6)$P_4$ in animal cells (Shears *et al.*, 1987; Balla *et al.*, 1987; Stephens *et al.*, 1988*a*), we have investigated the possibility that additional Ins$P_3$ isomers, acting as intermediates in currently undefined pathways of inositol polyphosphate metabolism, may be present in cells. The experiments reported below present evidence for the existence of four further [$^3$H]Ins$P_3$ isomers in [$^3$H]Ins-prelabelled avian erythrocytes. These additional Ins$P_3$ isomers may represent intermediates in uncharacterized pathways involved in Ins$P_5$ turnover. Although present at relatively low levels in avian erythrocytes, they could cause the basal levels of the previously defined inositol trisphosphates to be seriously overestimated as a consequence of their chromatographic similarity to these compounds.

## MATERIALS AND METHODS

### Preparation of avian erythrocytes and erythrocyte lysates

Blood was collected from 5-day-old chicks (approx. 1.5 ml per chick) and washed with iso-osmotic saline as described previously (Stephens *et al.*, 1988*a*). Erythrocytes (0.3 ml packed volume) were incubated with [$^3$H]Ins [1 mCi/ml of suspension in 1 ml of Dulbecco's modified Eagle's Medium containing 25 mM-Hepes and 5 % chicken serum (Gibco Ltd.) as described previously (Stephens *et al.*, 1988*a*)].

Centrifugally packed, washed erythrocytes were lysed by dilution into 5 vol. of ice-cold 5 mM-$MgCl_2$/5 mM-potassium phosphate/1 mM-EDTA/15 mM-2-mercaptoethanol/0.1 mM-phenylmethanesulphonyl fluoride (PMSF)/1 $\mu$g each of antipain, pepstatin A and leupeptin/ml. After 15 min on ice the lysate was used directly in assays.

Acid extracts of avian erythrocytes were prepared, neutralized, mixed with $^{32}$P standards (see below), applied to either Partisal 10-SAX or Partisphere 5-WAX anion-exchange h.p.l.c. columns (Jones Chromatography, Hengoed, Mid-Glamorgan, Wales, U.K., and Whatman respectively) and eluted using the buffers and gradients described previously (Stephens *et al.*, 1988*c*); Ins$P_3$ isomers were eluted from the Partisphere WAX column with 50 mM-$(NH_4)_2HPO_4$ (pH 3.2 with $H_3PO_4$, 25 °C).

[$^3$H]Phospholipids were extracted from avian erythrocytes by mixing the cellular debris produced during the preparation of an acid extract (see above) with 2 ml of $CHCl_3$/methanol/0.1 M-HCl (6:10:4, by vol.). The insoluble material was pelleted by centrifugation and the supernatant was partitioned by the addition of a further 0.526 ml of 0.1 M-HCl and chloroform. The lower phospholipid-rich phase was further washed, dried and deacylated exactly as described (Stephens *et al.*, 1989).

Fractions of h.p.l.c. eluate were desalted as described previously (Stephens *et al.*, 1988*c*).

### Preparation of inositol phosphates

Ins[$^{32}$P](1,4,5)$P_3$ was prepared from human erythrocytes as described previously (Hawkins *et al.*, 1986).

Ins[$^{32}$P](4,5,6)$P_3$ was prepared by incubation of

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Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; BSA, bovine serum albumin. The positions of phosphates on a given inositol phosphate are denoted by numbering from the position of the phosphate in D-Ins1$P$, unless otherwise stated.

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Ins[$^{32}$P](3,4,5,6)$P_4$ (Stephens *et al.*, 1988*c*) in a solution containing 15 units of alkaline phosphatase/ml (units as defined by Sigma; type P5521)/0.1 mM-ZnCl$_2$/0.1% (w/v) bovine serum albumin (BSA)/10 mM-ethanolamine (pH 9.5, 25 °C). Under these conditions optimum yields of Ins[$^{32}$P](4,5,6)$P_3$ were obtained after 10 min incubation. The reactions were quenched with HClO$_4$ (4%, v/v, final), neutralized with tri-n-octylamine/Freon (1:1, v/v; Sharpes & McCarl, 1982) and filtered before application to a Partisphere 5-SAX anion-exchange h.p.l.c. column as described previously (Stephens *et al.*, 1988*c*). The h.p.l.c. column was eluted with a gradient of water (buffer A) and 1.25 M-(NH$_4$)$_2$HPO$_4$ (pH 3.8 with H$_3$PO$_4$; 25 °C; buffer B) as follows: 0 min, 0% B; 12 min, 0% B; 25 min, 8% B; 52 min, 12% B; 53 min, 17% B; 87 min, 23% B; 107 min, 100% B; 112 min, 100% B; 113 min, 0% B. The flow rate was 1 ml·min$^{-1}$. Two Ins[$^{32}$P]$P_3$ isomers were detected amongst the reaction products. The fractions containing the isomer with the longer retention time (obtained in 14% yield from the starting Ins[$^{32}$P]$P_4$) were pooled and desalted. The identity of this peak was confirmed by processing a sample of [$^3$H]Ins(3,4,5,6)$P_4$ (which had been prepared from *myo*-[2-$^3$H]inositol-labelled avian erythrocytes) in parallel; the equivalent [$^3$H]Ins$P_3$ peak yielded volatile $^3$H upon periodate oxidation.

### Dephosphorylation, oxidation and acid-catalysed phosphate migration of inositol phosphates

Human erythrocyte ghosts (prepared as described in Hawkins *et al.*, 1983) were used to dephosphorylate various preparations of inositol phosphates. Inositol phosphates were mixed with human erythrocyte ghosts in a solution containing 50 mM-Hepes/2 mM-EDTA/1 mM-MgCl$_2$/1 mg of BSA/ml (pH 7.0, 37 °C) at a ghost-derived protein concentration of approx. 2 mg/ml. Reactions were quenched with 25 vol. of ice-cold 70% HClO$_4$. Protein was pelleted by centrifugation and the supernatant was neutralized with tri-n-octylamine/Freon (see above) and mixed with 40 vol. of 0.1 M-EDTA (NaOH to pH 7.0).

Inositol phosphates were randomly dephosphorylated with 10 M-NH$_4$OH at 110 °C as described previously (Stephens *et al.*, 1988*b*).

Controlled migration of phosphates across the *cis*-related 1 and 2, and 2 and 3, hydroxyl groups of inositol phosphates was achieved by boiling desalted preparations of inositol phosphates in 100 $\mu$l of 1.0 M-HCl for 8 min. The reactions were quenched with 100 $\mu$l of 1.0 M-NaOH/50 mM-Tris, diluted 20-fold with water and desalted as described above. Under these conditions no significant phosphate migration across *trans*-related substitution sites occurred (Pizer & Ballou, 1959; L. R. Stephens, unpublished work; and see Fig. 8).

Inositol phosphates were oxidized with sodium periodate (0.1 M-sodium periodate, pH 4.5), reduced and dephosphorylated as described (Stephens *et al.*, 1988*a*).

The glycerol moieties of Gro$P$[$^3$H]Ins$P$ isomers were removed exactly as described by Brown & Stewart (1966).

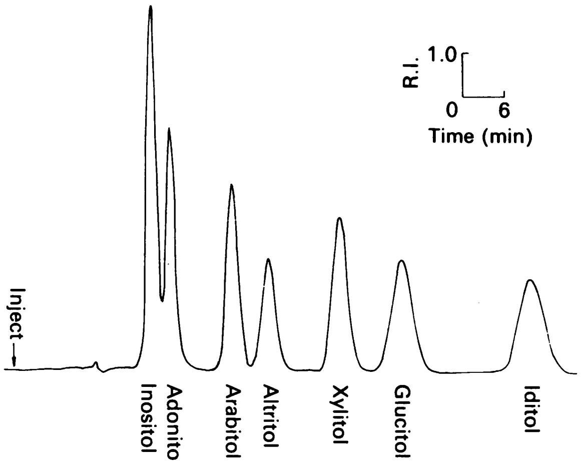

**Fig. 1. H.p.l.c. separation of the polyols that can be derived from [$^3$H]Ins$P_3$ isomers by periodate oxidation, reduction and dephosphorylation**

Water (10 $\mu$l) containing 20 $\mu$g each of *myo*-inositol, adonitol, arabitol, altritol, xylitol, glucitol and iditol was injected on to a cation-exchange h.p.l.c. column (in the Pb$^{2+}$ mode and held at 25 °C by a thermostated heating block; see the Materials and methods section) and eluted with water at 0.2 ml·min$^{-1}$. The relative refractive index (R.I.) of the column eluate was monitored continuously (Waters differential refractometer, 410). Pure solutions of the above polyols were injected on to the h.p.l.c. column to establish the identity of the peaks shown. All of the columns tested (with one exception out of ten) consistently reproduced the absolute retention times for the full range of polyols shown.

### Preparation and separation of polyols

The majority of polyols used were either prepared by reduction of their corresponding ketones with NaBH$_4$ or purchased from previously defined sources (Stephens *et al.*, 1988*a*). Tagatose and allulose were purchased from Sigma Chemical Co. L- and D-Altritol were prepared by reduction of L-talose and D-altrose respectively (obtained from Sigma). [$^{14}$C]Ins and [$^{14}$C]glucitol were purchased from Amersham International.

D-[$^{14}$C]Iditol was prepared from either Ptd[$^{14}$C]Ins-(4,5)$P_2$ as described previously (Stephens *et al.*, 1988*a*) or by partial periodate oxidation (12 h with 0.1 M-sodium periodate, pH 4.5, followed by reduction and dephosphorylation) of [$^{14}$C]Ins(1,4)$P_2$ (which was prepared from Ptd[$^{14}$C]Ins4$P$ as described in Stephens *et al.*, 1988*c*). L-[$^{14}$C]Altritol was prepared by partial periodate oxidation (as defined above) of [$^{14}$C]Ins(1,4)$P_2$; the products were reduced, dephosphorylated and purified (as described below). Typically, 7–10% and 45–55% of the radioactivity in the original [$^{14}$C]Ins(1,4)$P_2$ was recovered in L-[$^{14}$C]altritol and D-[$^{14}$C]iditol respectively. L-[$^3$H]-Altritol was prepared from [$^3$H]Ins(1,3,4)$P_3$ (which was itself prepared as described in Stephens *et al.*, 1988*c*).

Polyols were separated on a polypore-carbohydrate, cation-exchange h.p.l.c. column (in the Pb$^{2+}$ mode; Anachem Ltd.) as described previously (Stephens *et al.*, 1988*a*) and detected and/or quantified in the column eluate by liquid scintillation counting of individual fractions (typically 10-drop fractions) or by on-line differential refractometry (Waters, model 410 differential refractometer). Using this technique it was possible to resolve all of the polyols that can be derived from Ins$P_3$ isomers by periodate oxidation, reduction and dephosphorylation (see Fig. 1 and Table 1).

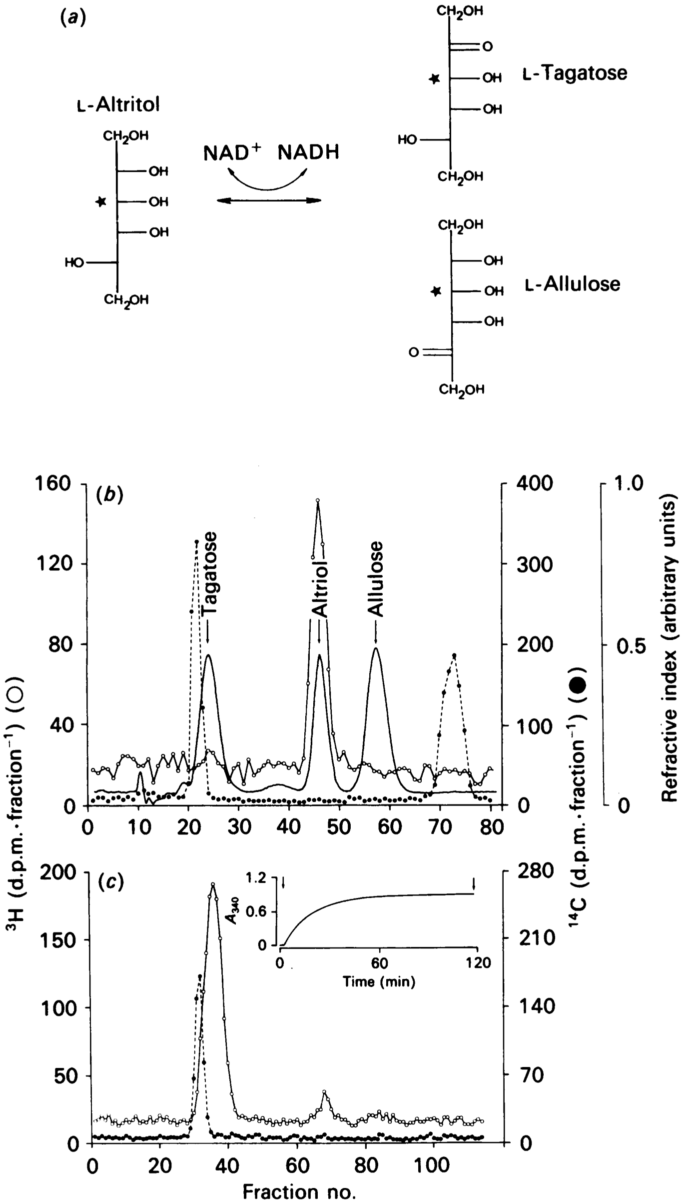

**Fig. 2. Oxidation of L-altritol by a commercially available, yeast-derived preparation of L-iditol dehydrogenase**

L-Altritol but not D-altritol is oxidized by a commercial preparation of L-iditol dehydrogenase (see the Materials and methods section). This oxidation could yield either tagatose and/or allulose depending on which end(s) of the polyol acts as a substrate for the enzyme; see (*a*). (*b*) and (*c*) show the results from an experiment in which a mixture containing [$^3$H]altritol derived from Ins(1,3,4)$P_3$ and 24 $\mu$g of L-altritol was incubated with yeast-derived L-iditol dehydrogenase (9.5 units/ml, conditions as defined above) for 0 (*b*) or 120 (*c*) min. The $A_{340}$ of the reaction mixture was monitored continuously (see inset in *c*), the incubation was terminated, desalted, the assay products were mixed with [$^{14}$C]Ins and/or [$^{14}$C]glucitol and resolved by h.p.l.c. The column eluate was collected into 55 s (12-drop) fractions which were individually counted for radioactivity utilizing standard dual-label liquid scintillation counting techniques. A refractive index trace from an independent separation, on the same h.p.l.c. column, of tagatose, altritol and allulose is also shown (*b*). The identity of the individual compounds was established by injecting them independently. * Position of $^3$H label.

### Incubation of polyols with polyol dehydrogenase preparations

Purified (see above) polyols were incubated with L-iditol dehydrogenase (either yeast- or sheep-liver-derived; Sigma) in assays of 1 ml total volume containing 100 mM-Tris/HCl (pH 8.3, 25 °C)/20 mM-$\beta$-NAD$^+$/1.5–9.5 units of polyol dehydrogenase/ml (units as defined by Sigma). Particulate matter was removed from the reconstituted enzyme preparation by centrifugation. Reactions were initiated in 1 ml quartz cuvettes by the addition of substrate. The progress of the reactions was monitored by measuring the absorbance of the solution at 340 nm in a water-cooled, dual-beam Perkin–Elmer spectrophotometer. The oxidation of L- and D-iditol by L-iditol dehydrogenase has been described previously (Stephens *et al.*, 1988*a*). The yeast-derived preparation of L-iditol dehydrogenase oxidized L-altritol at the C-2 position, yielding tagatose (see Fig. 2; oxidation at C-5 would have yielded allulose). The process had a $K_m$ with respect to L-altritol of 2 mM and a $V_{max.}$ of 35.1 nmol · min$^{-1}$ · unit$^{-1}$ (units as defined by Sigma). The first-order rate constant for the oxidation of L-altritol was measured to be 0.69 % · min$^{-1}$ · unit$^{-1}$, which was 11.3 % of that observed against L-iditol. D-Altritol was oxidized by the yeast-derived L-iditol dehydrogenase preparation with a first-order rate constant of 0.0052 % · min$^{-1}$ · unit$^{-1}$ (i.e. $\frac{1}{132}$ of that observed against L-altritol under the same conditions). The first-order rate constant for the oxidation of L-iditol by a commercially available sheep liver-derived L-iditol dehydrogenase preparation (Sigma) was 12.6 % · min$^{-1}$ · unit$^{-1}$; that for L-altritol was $\frac{1}{330}$ of this value. Whether this represents a species difference in the substrate specificity of L-iditol dehydrogenase or the presence of more than one enzyme in the yeast L-iditol dehydrogenase preparation has not been established. Routinely, assays contained either 100–200 $\mu$M-L-iditol and 1.5 units of yeast-derived L-iditol dehydrogenase/ml (see Stephens *et al.*, 1988*a*) or 100–200 $\mu$M-L-altritol and 9.5 units of yeast-derived dehydrogenase/ml. Altritol oxidations were usually run for 120–150 min, then quenched by heating the assay mixture to 100 °C for 3 min. The solution was deionized with 2 ml of mixed-bed ion-exchange resin (MB3; Sigma) dried by lyophilization, resuspended in 10 $\mu$l of water and reapplied to a cation-exchange h.p.l.c. column (in the Pb$^{2+}$ mode) to separate and quantify the radioactive reactants and products. The precise extent of the reaction was determined by measuring the proportion of internal L-[$^{14}$C]altritol oxidized to [$^{14}$C]tagatose. It is not possible to assess the proportion of L-altritol oxidized to tagatose during one of these assays by the same strategy as that utilized to quantify L-iditol oxidations (i.e. by best-fitting the $A_{340}$ curve describing NADH production to an exponential which is then extrapolated to yield a 100 % value: the extent of reaction at time *t* being calculated from the observed $A_{340}$ as a proportion of the predicted maximum $A_{340}$; see Stephens *et al.*, 1988*a*), as the rate of destruction of NADH during the incubation was such that by the time the reaction was terminated a significant proportion had been lost.

### Gel-filtration chromatography of rat brain cytosol

Brains from 200 g male rats were homogenized in 0.25 M-sucrose/50 mM-Hepes/2 mM-EGTA/15 mM-2-mercaptoethanol/0.1 mM-PMSF and 1 $\mu$g/ml each of antipain, pepstatin A and leupeptin (pH 7.0, 4 °C) as described previously (Stephens *et al.*, 1988*c*). A 20–50 % (% saturation at 4 °C) $(NH_4)_2SO_4$ fraction was prepared from a 100000 *g* supernatant as described previously (Stephens *et al.*, 1988*c*). The precipitated protein was redissolved in approx. 2 ml of 0.5 M-KCl/50 mM-Hepes/2 mM-EGTA/15 mM-2-mercaptoethanol/0.1 mM-PMSF/1 $\mu$g of antipain, leupeptin and pepstatin A/ml (to approx. 20 mg of protein/ml) and dialysed against 1 litre of the same buffer for 2 h. A sample of 100 $\mu$l of the dialysed preparation was immediately applied to a calibrated gel-filtration column (Superose-12 f.p.l.c. column; Pharmacia) which had been pre-equilibrated with the dialysis buffer described above. The column was eluted at 0.3 ml·min$^{-1}$ into 0.5 ml fractions from the $V_0$ (7.5 ml) to the $V_t$ (19.5 ml).

### Assay of [$^3$H]Ins(1,3,4,6)$P_4$ and [$^3$H]Ins(3,4,5,6)$P_4$ dephosphorylation

Aliquots (250 $\mu$l) from each of the fractions collected from the gel-filtration column were assayed for [$^3$H]Ins(1,3,4,6)$P_4$ and [$^3$H]Ins(3,4,5,6)$P_4$ phosphomonoesterase activities in a buffer of final composition 50 mM-Hepes/2 mM-EGTA/15 mM-2-mercaptoethanol/0.2 M-KCl/1 mM-$MgCl_2$/1 mg of BSA/ml (pH 7.0, 37 °C) and 4000 d.p.m. of either [$^3$H]Ins(1,3,4,6)$P_4$ or [$^3$H]Ins(3,4,5,6)$P_4$ (prepared as described previously; Stephens *et al.*, 1988*c*). The substrates were present at concentrations of approx. 5 and 50 nM respectively, in a total assay volume of 2 ml. The reactions were quenched with 2 ml of ice-cold 10 % (v/v) $HClO_4$, which was subsequently removed with tri-n-octylamine/Freon as described above and mixed with 50 $\mu$l of 0.1 M-EDTA (pH 7.0, 25 °C).

## RESULTS

Acid extracts from [$^3$H]Ins-prelabelled avian erythrocytes were prepared for application to an anion-exchange h.p.l.c. column and mixed with [$^{32}$P]ATP, Ins[$^{32}$P](1,4,5)$P_3$ and Ins[$^{32}$P](4,5,6)$P_3$. The [$^3$H]Ins$P_3$ isomers in the extract were resolved with either a Partisil 10-SAX anion-exchange h.p.l.c. column (eluted as described in Stephens *et al.*, 1988*a*) or a Partisphere 5-WAX column eluted as described in the Materials and methods section.

In a typical preparation of [$^3$H]Ins-prelabelled avian erythrocytes, 1–2 $\mu$Ci of [$^3$H]Ins$P_3$ isomers, 4–7 $\mu$Ci of [$^3$H]Ins$P_4$ isomers and 7–12 $\mu$Ci of [$^3$H]Ins$P_5$ were recovered. Two peaks of $^3$H were eluted from a Partisil 10-SAX h.p.l.c. column at times expected for Ins$P_3$ isomers. The earliest eluting [$^3$H]Ins$P_3$(s) possessed a retention time very close to that of [$^{32}$P]ATP; the second [$^3$H]Ins$P_3$(s) eluted with the Ins[$^{32}$P](4,5,6)$P_3$ and Ins[$^{32}$P](1,4,5)$P_3$ standards (results not shown). Three peaks of $^3$H designated I, II and III in order of increasing retention time were eluted from a weak anion-exchange h.p.l.c. column (see Fig. 3). Ins[$^{32}$P](1,4,5)$P_3$ eluted with the second $^3$H peak (although the $^3$H reproducibly eluted just before the $^{32}$P). Ins[$^{32}$P](4,5,6)$P_3$ eluted considerably later than any of the [$^3$H]Ins$P_3$s, and [$^{32}$P]ATP eluted close to the first of the [$^3$H]Ins$P_3$ peaks (peak I).

Fractions comprising peak I, peak II and peak III [$^3$H]Ins$P_3$ isomers were pooled and desalted as described above.

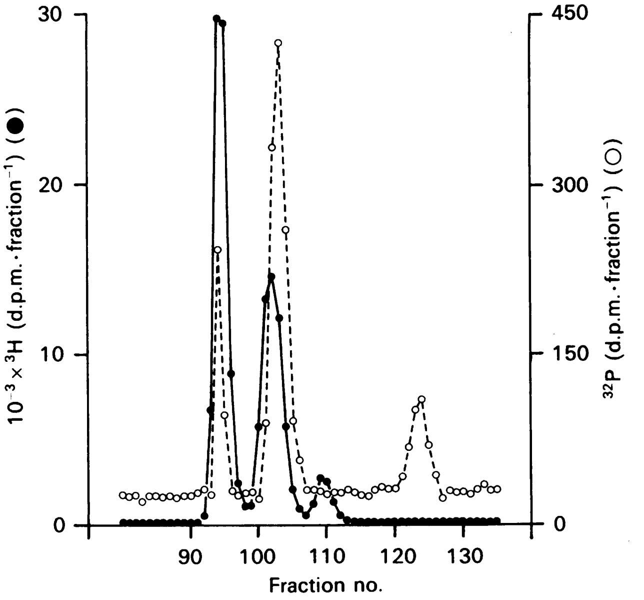

**Fig. 3. Separation of [$^3$H]Ins$P_3$ isomers in an acid extract of [$^3$H]Ins-prelabelled avian erythrocytes by anion-exchange h.p.l.c.**

An acid extract was prepared from [$^3$H]Ins-prelabelled erythrocytes from 5-day-old chicks as described. The neutralized sample was mixed with Ins[$^{32}$P](1,4,5)$P_3$, [$^{32}$P]ATP and Ins[$^{32}$P](4,5,6)$P_3$ (prepared or purchased from sources defined in the Materials and methods section), applied to a weak anion-exchange h.p.l.c. column, and eluted into 0.4 min fractions which were individually counted for radioactivity utilizing standard dual-label liquid scintillation counting techniques. The data shown focus on a window in the chromatographic profile in which the $^{32}$P standards were eluted. In three further independent preparations of [$^3$H]Ins$P_3$s, three peaks of $^3$H radioactivity were always resolved, although there was some variation in the relative sizes of the peaks recovered. The peaks of $^3$H were designated [$^3$H]Ins$P_3$(s) I, II and III in order of increasing retention time. The fractions containing a particular peak were pooled and desalted as described.

### Identification of [$^3$H]Ins$P_3$(s) in peak I

A portion of the peak I Ins$P_3$(s) was oxidized with 0.1 M-sodium periodate then reduced and dephosphorylated as described. A total of 88 % of the starting $^3$H radioactivity was recovered in [$^3$H]altritol. The only Ins$P_3$s that can yield altritol are D- or L-Ins(1,3,4)$P_3$ or D- or L-Ins(1,2,4)$P_3$ (see Table 1). A second portion of the desalted preparation of peak I [$^3$H]Ins$P_3$s was dephosphorylated with 10 M-$NH_4OH$. The sample was mixed with D- and L-[$^{14}$C]Ins1$P$, [$^{14}$C]Ins2$P$ and [$^{14}$C]Ins4$P$ (prepared as described in Stephens *et al.*, 1988*c*) and resolved on a Partisphere 5-SAX anion-exchange h.p.l.c. column [eluted isocratically with 5 mM-$(NH_4)_2HPO_3$, pH 4.6/$H_3PO_4$, 25 °C; results not shown]. 0.8 % of the total $^3$H recovered from the hydrolysate was in D- or L-[$^3$H]Ins1$P$ and D- or L-[$^3$H]Ins4$P$; no $^3$H radioactivity could be detected that co-eluted with [$^{14}$C]Ins2$P$. This suggests that the only [$^3$H]Ins$P_3$ isomers that can be present in peak I are D- or L-[$^3$H]Ins(1,3,4)$P_3$.

[$^3$H]-Altritol derived from the peak I [$^3$H]Ins$P_3$(s) was

**Table 1. The structures, and the polyols that would be produced from them, of all the possible non-cyclic isomers of *myo*-inositol trisphosphate**

D-Glucitol ≃ sorbitol; adonitol ≃ ribitol. L-Iditol dehydrogenase has been used to distinguish between D- and L-iditol and D- and L-altritol. D- and L-Arabitol and D- and L-glucitol can also be discriminated by this preparation (although xylitol and adonitol are also substrates for this enzyme, their optical inactivity renders the oxidation reaction impotent in terms of structural information about the inositol phosphates from which they were originally derived). Phosphates in inositol trisphosphate isomers are numbered from the D-1 substitution site of *myo*-inositol throughout.

| Inositol trisphosphate | Enantiomer of that Ins$P_3$ | Polyol derived from that Ins$P_3$ |
|---|---|---|
| Ins(1,2,3)$P_3$ | *meso* | Adonitol* |
| Ins(1,2,4)$P_3$ | Ins(2,3,6)$P_3$ | L-Altritol |
| Ins(1,2,5)$P_3$ | Ins(2,3,5)$P_3$ | D-Glucitol |
| Ins(1,2,6)$P_3$ | Ins(2,3,4)$P_3$ | D-Arabitol |
| Ins(1,3,4)$P_3$ | Ins(1,3,6)$P_3$ | L-Altritol |
| Ins(1,3,5)$P_3$ | *meso* | Ins* |
| Ins(1,3,6)$P_3$ | Ins(1,3,4)$P_3$ | D-Altritol |
| Ins(1,4,5)$P_3$ | Ins(3,5,6)$P_3$ | D-Iditol |
| Ins(1,4,6)$P_3$ | Ins(3,4,6)$P_3$ | D-Iditol |
| Ins(1,5,6)$P_3$ | Ins(3,4,5)$P_3$ | Xylitol* |
| Ins(2,3,4)$P_3$ | Ins(1,2,6)$P_3$ | L-Arabitol |
| Ins(2,3,5)$P_3$ | Ins(1,2,5)$P_3$ | L-Glucitol |
| Ins(2,3,6)$P_3$ | Ins(1,2,4)$P_3$ | D-Altritol |
| Ins(2,4,5)$P_3$ | Ins(2,5,6)$P_3$ | L-Glucitol |
| Ins(2,4,6)$P_3$ | *meso* | Ins* |
| Ins(2,5,6)$P_3$ | Ins(2,4,5)$P_3$ | D-Glucitol |
| Ins(3,4,5)$P_3$ | Ins(1,5,6)$P_3$ | Xylitol* |
| Ins(3,4,6)$P_3$ | Ins(1,4,6)$P_3$ | L-Iditol |
| Ins(3,5,6)$P_3$ | Ins(1,4,5)$P_3$ | D-Iditol |
| Ins(4,5,6)$P_3$ | *meso* | Xylitol*† |

* Optically inactive polyol.

† If Ins(4,5,6)$P_3$ is labelled with *myo*-[2-$^{3}$H]inositol, then upon periodate oxidation the $^{3}$H is lost.

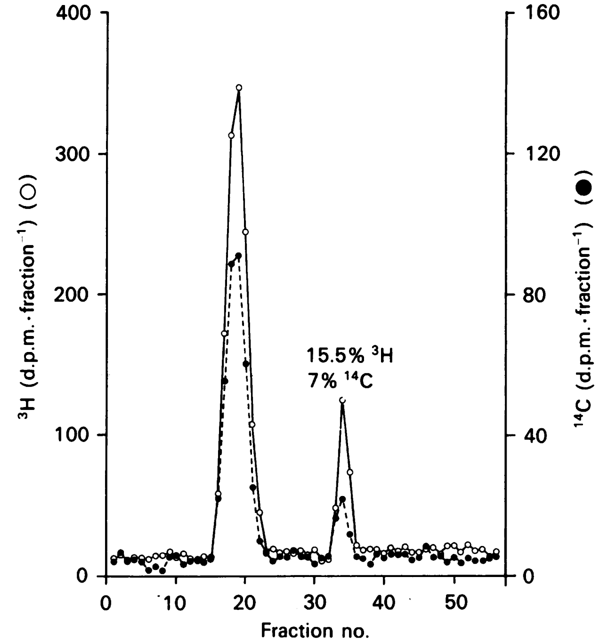

**Fig. 4. L-Iditol dehydrogenase-catalysed oxidation of L-[$^{14}$C]-altritol and [$^{3}$H]altritol that was derived from avian erythrocyte peak I [$^{3}$H]Ins$P_3$(s)**

The [$^{3}$H]altritol, derived from the periodate oxidation, reduction and dephosphorylation of avian erythrocyte peak I [$^{3}$H]Ins$P_3$(s) was mixed with L-[$^{14}$C]altritol and 20 μg of L-altritol and incubated with a commercially available yeast-derived preparation of L-iditol dehydrogenase (9.5 units/ml, conditions as defined above) for 0 min (results not shown) or 130 min. The reaction was quenched when greater than 85 % of L-altritol had been oxidized (as judged by the quantity of NADH generated during the assay). The reaction was terminated, desalted and reapplied to a cation-exchange h.p.l.c. column (in the Pb$^{2+}$ mode, see the Materials and methods section); 12-drop fractions (approx. 0.93 min) were collected and individually counted for $^{3}$H and $^{14}$C radioactivity utilizing standard dual-label liquid scintillation counting techniques. The 0 min control sample contained single peaks of $^{3}$H and $^{14}$C which eluted at the time expected for altitrol (results not shown). The proportions (as a % of the total of each isotope recovered) of the L-[$^{14}$C]altritol and [$^{3}$H]altritol remaining at the end of the assay are defined in the Figure.

mixed with L-[$^{14}$C]altritol and incubated with L-iditol dehydrogenase (yeast-derived; see above). The assay mixture was desalted and reapplied to a 'carbohydrate'-h.p.l.c. column (see Fig. 4 and the Materials and methods section); 93 and 84.5 % of the L-[$^{14}$C]altritol and [$^{3}$H]altritol respectively had been oxidized to [$^{14}$C]- and [$^{3}$H]tagatose. Hence the [$^{3}$H]Ins$P_3$(s) in peak I were [$^{3}$H]Ins(1,3,4)$P_3$ (91 % of the total) and [$^{3}$H]Ins(1,3,6)$P_3$ (9 % of the total; see Table 1 and below for discussion). Three independent preparations of the [$^{3}$H]Ins$P_3$(s) fraction contained between 5 and 12 % [$^{3}$H]Ins(1,3,6)$P_3$.

### Identification of [$^{3}$H]Ins$P_3$(s) in peak II

An aliquot of the desalted preparation of peak II [$^{3}$H]Ins$P_3$(s) was oxidized with sodium periodate, reduced and dephosphorylated. The [$^{3}$H]polyols recovered (79 % of the radioactivity in the starting [$^{3}$H]inositol phosphates) were separated on an h.p.l.c. column as described above. The only significant product was [$^{3}$H]-iditol (96 % of the $^{3}$H recovered) which could arise from any of the four Ins$P_3$ isomers: D- or L-[$^{3}$H]Ins(1,4,5)$P_3$ and D- or L-[$^{3}$H]Ins(1,4,6)$P_3$ (see Table 1).

Portions of the [$^{3}$H]iditol which was derived from peak II [$^{3}$H]Ins$P_3$(s) were incubated with L-iditol dehydrogenase (as described above) for various times. The proportion of [$^{3}$H]iditol oxidized to [$^{3}$H]sorbose was determined at each time and extrapolated to yield an estimate of the total L-[$^{3}$H]iditol in the preparation (19.3 %; see Fig. 5). This suggests that 19.3 % of the peak II [$^{3}$H]Ins$P_3$s are [$^{3}$H]Ins(3,5,6)$P_3$ and/or [$^{3}$H]Ins(3,4,6)$P_3$ (see Table 1).

A second portion of peak II was dried down, dissolved in 0.1 M-HCl and heated to 100 °C for 8 min. The sample was neutralized and desalted [conditions which catalyse migration of phosphates between *cis*-related, but not *trans*-related, hydroxyl moieties in *myo*-inositol (Pizer & Ballou, 1959); see Fig. 8] before being oxidized with sodium periodate and processed as defined for peak I above. Of the radioactivity in the [$^{3}$H]inositol phosphate(s), 62 % was recovered in the following polyols: [$^{3}$H]inositol, 10 % (of the total recovered [$^{3}$H]-

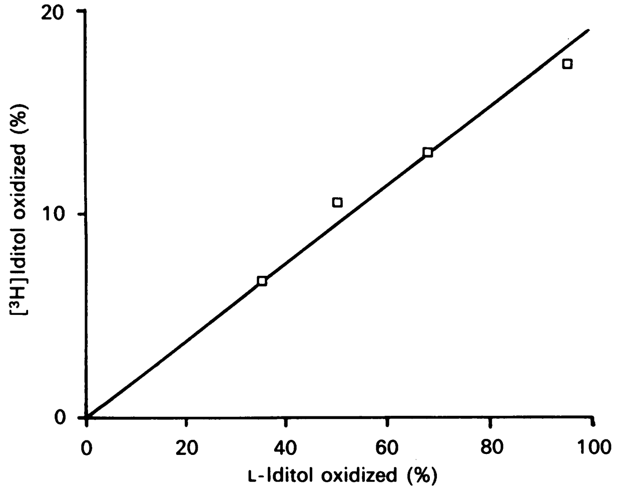

**Fig. 5. Oxidation of [$^{3}$H]iditol, derived from avian erythrocyte [$^{3}$H]Ins$P_3$ isomers, by a commercially available yeast-derived preparation of L-iditol dehydrogenase**

[$^{3}$H]Iditol derived from the periodate oxidation of avian erythrocyte peak II [$^{3}$H]Ins$P_3$(s) and approx. 20 $\mu$g of L-iditol were incubated with 1.5 units of yeast-derived L-iditol dehydrogenase/ml for various times. The reactions were quenched and desalted, as described, and the proportion of the L-iditol oxidized at each time was estimated from the $A_{340}$ curves describing the production of NADH during the progress of the assay (precisely as described in Stephens *et al.*, 1988*a*). The products were resolved with a cation-exchange h.p.l.c. column (in the $Pb^{2+}$ mode) and the $^{3}$H-labelled metabolites were quantified by liquid scintillation counting as described. The data from several such experiments are presented as a series of points each defining the proportions of both L-iditol and [$^{3}$H]iditol oxidized in a particular assay. They extrapolated, at 100% L-iditol oxidation, to $19.3 \pm 0.6$% (mean ± S.E.M., $n = 4$) of the [$^{3}$H]iditol being oxidized.

polyol); [$^{3}$H]xylitol, 10.5%; [$^{3}$H]glucitol, 29.5%; and [$^{3}$H]iditol, 45%. This result is consistent with peak II [$^{3}$H]Ins$P_3$ containing both D- or L-[$^{3}$H]Ins(1,4,5)$P_3$ and D- or L-[$^{3}$H]Ins(1,4,6)$P_3$. The distribution of radioactivity amongst the polyols suggests that the major [$^{3}$H]Ins$P_3$(s) in this fraction is D- or L-[$^{3}$H]Ins(1,4,5)$P_3$.

A third portion of peak II was incubated with an internal 'spike' of Ins[$^{32}$P](1,4,5)$P_3$ and human erythrocyte ghosts (as described in the Materials and methods section). After various times, the assays were terminated and the products resolved on small Bio-Rad AG 1 × 8 (200–400, formate form) columns (see above and Fig. 6). The Ins[$^{32}$P](1,4,5)$P_3$ standard was completely converted to Ins[$^{32}$P]$P_2$ and [$^{32}$P]$P_i$, but 28% of the total [$^{3}$H]Ins$P_3$(s) present was resistant to dephosphorylation by erythrocyte ghosts.

A scaled up version of the above assay was used to prepare a quantity of the erythrocyte-ghost-resistant [$^{3}$H]Ins$P_3$(s) found in peak II. The erythrocyte-ghost-resistant [$^{3}$H]Ins$P_3$ was purified by anion-exchange h.p.l.c. (on a Partisil 10-SAX column) and desalted. A portion of this sample was oxidized with sodium periodate, reduced and dephosphorylated. Some 86% of the radioactivity in the [$^{3}$H]inositol phosphates was recovered as [$^{3}$H]iditol with no other [$^{3}$H]polyol being detected. When this was incubated with L-iditol dehydrogenase (see above and Fig. 7), 98% of the internal L-iditol was oxidized whilst 72% of the initial [$^{3}$H]iditol was oxidized to [$^{3}$H]sorbose, suggesting that 74% of the human erythrocyte-ghost-resistant peak II [$^{3}$H]Ins$P_3$ was [$^{3}$H]Ins(3,4,6)$P_3$ and/or [$^{3}$H]Ins(3,5,6)$P_3$ (see Table 1) and that 24% was [$^{3}$H]Ins(1,4,6)$P_3$.

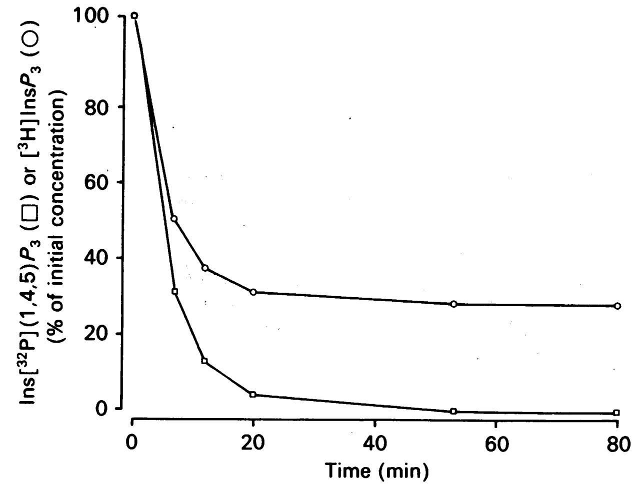

**Fig. 6. Dephosphorylation of avian erythrocyte-derived peak II [$^{3}$H]Ins$P_3$ isomers with human erythrocyte ghosts**

Aliquots of avian erythrocyte-derived, h.p.l.c.-purified, desalted peak II [$^{3}$H]Ins$P_3$s were mixed with Ins[$^{32}$P](1,4,5)$P_3$ and human erythrocyte ghosts before being incubated (under the conditions defined in the Materials and methods section) for various times. Reactions were quenched, processed for application to small 'open' columns of Bio Rad AG 18 anion-exchange resin (200–400, in the formate form) and resolved by elution from the columns. The mean ($n = 2$) proportions of Ins[$^{32}$P](1,4,5)$P_3$ (□) and [$^{3}$H]Ins$P_3$(s) (○) remaining at various times are presented. The assays all contained 10800 d.p.m. of Ins[$^{32}$P](1,4,5)$P_3$ and 20050 d.p.m. of [$^{3}$H]Ins$P_3$(s). 28% of the [$^{3}$H]Ins$P_3$(s) remained unmetabolized even though all of the Ins[$^{32}$P](1,4,5)$P_3$ had been metabolized to [$^{32}$P]$P_i$ and Ins[$^{32}$P]$P_2$. In a preparative experiment {20-fold increase in the quantity of all the reagents added except for Ins[$^{32}$P](1,4,5)$P_3$} the reaction was quenched after 90 min and after appropriate processing, the products were resolved on an anion-exchange h.p.l.c. column (Partisil 10-SAX eluted as described above). 27.5% of the total $^{3}$H radioactivity recovered eluted at a time expected for a [$^{3}$H]Ins$P_3$; no Ins[$^{32}$P](1,4,5)$P_3$ remained (results not shown). The fractions containing the residual [$^{3}$H]Ins$P_3$ were pooled and desalted as described.

A second portion of ghost-resistant peak II [$^{3}$H]Ins$P_3$ was boiled with 1.0 M-HCl for 8 min (conditions which catalyse the migration of phosphates between *cis*-related, but not *trans*-related, hydroxyl moieties in *myo*-inositol), neutralized, desalted, oxidized with sodium periodate, reduced and dephosphorylated (see the Materials and methods section and Fig. 8; 70% of the original $^{3}$H radioactivity contained in the [$^{3}$H]Ins$P_3$(s) was recovered in [$^{3}$H]polyols). In this case, 38% of recovered $^{3}$H co-migrated with [$^{14}$C]Ins and 57% had the chromatographic mobility expected of [$^{3}$H]iditol. This suggests that the major human erythrocyte-ghost-resistant peak II [$^{3}$H]Ins$P_3$s are [$^{3}$H]Ins(1,4,6)$P_3$ and/or [$^{3}$H]Ins(3,4,6)$P_3$. This result was confirmed by an experiment in which an

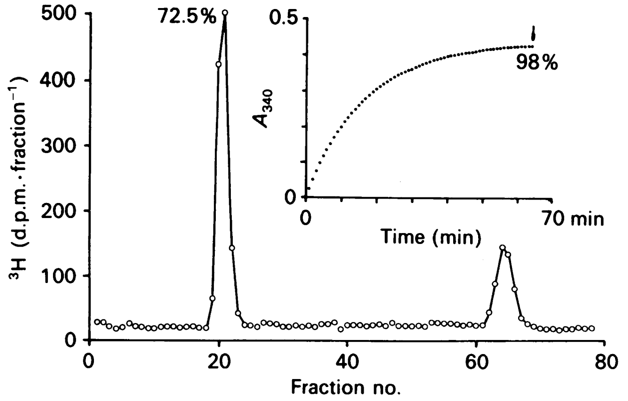

**Fig. 7. L-Iditol dehydrogenase-catalysed oxidation of D-[$^{14}$C]-iditol and of the [$^{3}$H]iditol which was generated from avian erythrocyte-derived, erythrocyte-ghost-resistant peak II [$^{3}$H]Ins$P_3$(s)**

The [$^{3}$H]iditol derived from the periodate oxidation, reduction and dephosphorylation of avian-erythrocyte-derived, human-erythrocyte ghost-resistant peak II [$^{3}$H]Ins$P_3$(s) was mixed with D-[$^{14}$C]iditol and 20 $\mu$g of L-iditol and incubated for 0 or 65 min with a commercially available yeast-derived preparation of L-iditol dehydrogenase (1.5 units/ml) under the conditions defined in the Materials and methods section. The reactions were terminated, desalted and the products resolved on a cation-exchange h.p.l.c. column (in the Pb$^{2+}$ mode) as described. The proportion of L-iditol oxidized after 90 min was estimated from the production of NADH (see inset). The proportions of D-[$^{14}$C]iditol and [$^{3}$H]iditol that had been simultaneously oxidized were assessed by individually counting, utilizing standard dual-label liquid scintillation counting techniques, 12-drop fractions of column eluant. The zero-time control contained a single peak of $^{3}$H and $^{14}$C radioactivity which eluted at the time anticipated for iditol. The proportions (as a % of the total of each of the isotopes recovered from the assays) of each of the substrates oxidized are shown in the Figure.

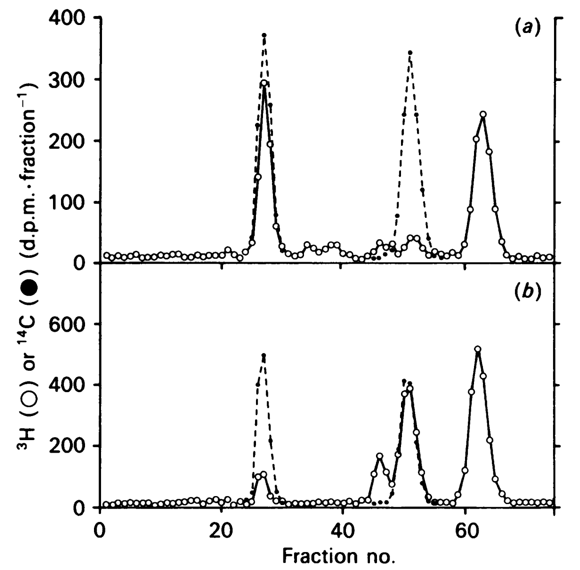

**Fig. 8. Acid-catalysed phosphate migration in [$^{3}$H]Ins(1,4,5)$P_3$ and avian-erythrocyte-derived, human-erythrocyte-ghost-resistant peak II [$^{3}$H]Ins$P_3$ (s)**

Aliquots of [$^{3}$H]Ins(1,4,5)$P_3$ (*b*) and avian erythrocyte-derived, human erythrocyte ghost-resistant peak II [$^{3}$H]Ins$P_3$(s) (*a*, see Fig. 6) were dissolved in 1.0 M-HCl and boiled. After 8 min the samples were cooled, neutralized (control samples were neutralized before being boiled), and processed to obtain polyols. The [$^{3}$H]polyols so derived were mixed with [$^{14}$C]Ins and [$^{14}$C]glucitol, resolved on a cation-exchange h.p.l.c. column (in the Pb$^{2+}$ mode) and quantified as described above. Control [$^{3}$H]Ins(1,4,5)$P_3$ samples contained a single peak of $^{3}$H which eluted at the time expected for iditol (results not shown). The control for the avian erythrocyte [$^{3}$H]Ins$P_3$(s) similarly contained a single peak of $^{3}$H which eluted at the time expected for iditol.

aliquot of ghost-resistant peak II [$^{3}$H]Ins$P_3$ was mixed with Ins[$^{32}$P](1,4,5)$P_3$, applied to a Partisphere 5-WAX anion-exchange h.p.l.c. column and eluted as described in the Materials and methods section (see Fig. 9). The [$^{3}$H]Ins$P_3$ isomers present in this extract were clearly retained less by the column than the $^{32}$P standard, suggesting the major [$^{3}$H]Ins$P_3$s present were neither D- nor L-[$^{3}$H]Ins(1,4,5)$P_3$ (see Table 1).

Taken together, these results suggest the original peak II [$^{3}$H]Ins$P_3$ contained 72 % [$^{3}$H]Ins(1,4,5)$P_3$ (rapidly metabolized by the erythrocyte ghosts, yielding D-[$^{3}$H]-iditol, and [$^{3}$H]glucitol and [$^{3}$H]xylitol when the sample was pretreated with acid), 20 % [$^{3}$H]Ins-(3,4,6)$P_3$ (resistant to the erythrocyte ghosts, yielding L-[$^{3}$H]iditol and some of the additional [$^{3}$H]Ins if the sample was pretreated with acid) and 8 % [$^{3}$H]Ins(1,4,6)$P_3$ (resistant to the human erythrocyte ghosts, yielding D-[$^{3}$H]iditol and some of the additional [$^{3}$H]Ins if the sample was pretreated with acid).

If any [$^{3}$H]Ins(3,5,6)$P_3$ had been present in peak II [$^{3}$H]Ins$P_3$s then it would have yielded L-[$^{3}$H]iditol upon periodate oxidation, reduction and dephosphorylation (see Table 1). Because all of the [$^{3}$H]Ins$P_3$s that gave L-[$^{3}$H]iditol were resistant to human erythrocyte ghosts and the erythrocyte-ghost-resistant [$^{3}$H]Ins$P_3$s were very largely D- and L-[$^{3}$H]Ins(1,4,6)$P_3$ then, within the error of these observations, the original peak II [$^{3}$H]Ins$P_3$s can be stated to contain no [$^{3}$H]Ins(3,5,6)$P_3$.

The above assumption, that all of the L-[$^{3}$H]iditol-yielding [$^{3}$H]Ins$P_3$(s) were resistant to hydrolysis in the presence of erythrocyte ghosts, is based on the following analysis. Some 19.3 % of the [$^{3}$H]iditol derived from the original peak II [$^{3}$H]Ins$P_3$s gave L-[$^{3}$H]iditol, whereas 74 % of the erythrocyte-ghost-resistant [$^{3}$H]Ins$P_3$s (which constituted 28 % of the total [$^{3}$H]Ins$P_3$s) transformed to L-[$^{3}$H]iditol. The expected value, if none of the [$^{3}$H]Ins$P_3$s yielding L-iditol were dephosphorylated by erythrocyte ghosts, of 69 %, suggests that, within the error of the experiments, the assumption is likely to be correct. The only possible anomaly would be if the erythrocyte-ghost-'resistant' [$^{3}$H]Ins$P_3$s that yield D-[$^{3}$H]iditol are actually dephosphorylated at a low but identical relative rate to the [$^{3}$H]Ins$P_3$s yielding L-[$^{3}$H]iditol.

### Identification of [$^{3}$H]Ins$P_3$ isomers in peak III

The [$^{3}$H]Ins$P_3$(s) in peak III (Fig. 3) yielded [$^{3}$H]xylitol upon periodate oxidation, reduction and dephosphorylation (75 % of the $^{3}$H radioactivity in the starting material was recovered in [$^{3}$H]polyol(s) of which 95 % was in [$^{3}$H]xylitol). The only [$^{3}$H]Ins$P_3$ (when $^{3}$H-labelled in the

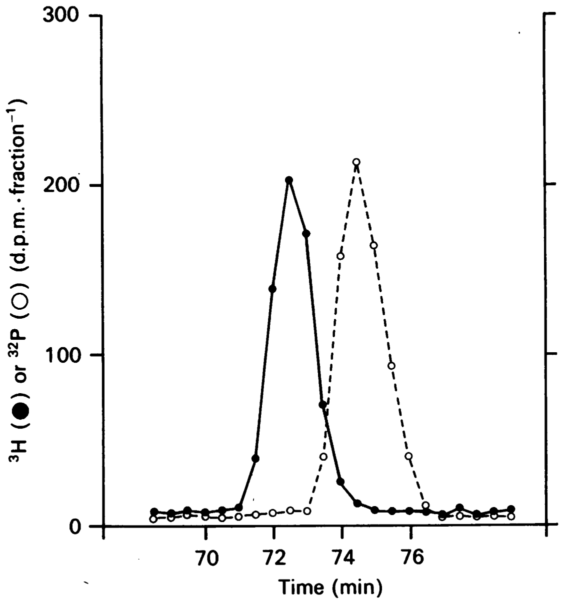

**Fig. 9. Separation of avian-erythrocyte-derived, erythrocyte-ghost-resistant peak II [$^3$H]Ins$P_3$(s) from Ins[$^{32}$P](1,4,5)$P_3$ by anion-exchange h.p.l.c.**

An aliquot of avian-erythrocyte-derived, erythrocyte-ghost-resistant peak II [$^3$H]Ins$P_3$(s) was mixed with Ins-[$^{32}$P](1,4,5)$P_3$ and applied to an anion-exchange h.p.l.c. column (Partisphere WAX h.p.l.c. column, eluted as described). Fractions were collected every 0.5 min and individually counted for $^{32}$P and $^3$H radioactivity by standard dual-label liquid scintillation counting techniques. The results shown were reproduced in two further experiments.

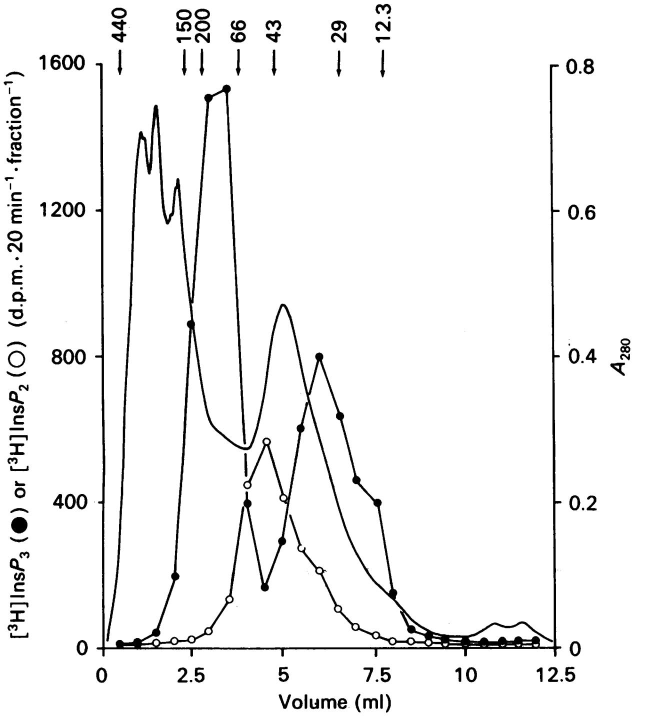

**Fig. 10. Gel-filtration chromatography of Ins(1,3,4,6)$P_4$ phosphomonoesterase activities from rat brain cytosol**

Protein from a 20–50 % $(NH_4)_2SO_4$ (% saturation at 4 °C) fraction of rat brain cytosol was prepared for gel-filtration chromatography as described in the Materials and methods section. An aliquot (100 $\mu$l) of the dialysed protein sample was injected on to a calibrated gel-filtration column which had been pre-equilibrated with sample buffer. The flow was 0.3 ml·min$^{-1}$ and the $A_{280}$ of the column eluate was monitored continuously. Fractions of 0.5 ml were collected from the $V_0$ (7.5 ml) to the $V_t$ (19.5 ml). Each fraction was assayed in duplicate for Ins(1,3,4,6)$P_4$ phosphomonoesterase activity as described. The data are presented as the mean $^3$H recovered from the assays in either [$^3$H]Ins$P_2$ (○) or [$^3$H]Ins$P_3$ (●) after 20 min incubation. A maximum of 40 % of the starting substrate was hydrolysed. Five similar experiments reproducibly resolved two peaks of phosphomonoesterase activity eluting at volumes suggesting native molecular masses of 31 and 150 kDa; however there was some variation between different preparations in the relative quantity of phosphomonoesterase activity recovered in these two peaks. Molecular mass markers were ferritin, 440 kDa; $\beta$-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa and cytochrome *c*, 12.3 kDa.

C-2 position of the inositol moiety) that can yield [$^3$H]xylitol upon periodate oxidation, reduction and dephosphorylation are D- or L-[$^3$H]Ins(1,5,6)$P_3$ (see Table 1). As xylitol is a *meso*-compound, no further information about the structure of the original inositol phosphate can be gained by study of the [$^3$H]xylitol.

### Identification of Ptd[$^3$H]Ins$P_2$(s) in chick erythrocytes

A phospholipid extract was prepared from [$^3$H]Ins-prelabelled avian erythrocytes and deacylated (as described above). The resulting Gro$P$[$^3$H]Ins$P_2$s were purified by anion-exchange h.p.l.c. (on a Partisil 10-SAX h.p.l.c. column), desalted and their glycerol moieties removed. The resulting [$^3$H]Ins$P_3$(s) co-chromatographed with Ins[$^{32}$P](1,4,5)$P_3$, yielded D-[$^3$H]iditol upon periodate oxidation, reduction and dephosphorylation and was dephosphorylated in parallel with an 'internal spike' of Ins[$^{32}$P](1,4,5)$P_3$ by human erythrocyte ghosts (results not shown). The results from these three independent experimental protocols all suggest that >98 % of the Ptd[$^3$H]Ins$P_2$ in [$^3$H]Ins-prelabelled avian erythrocytes is Ptd[$^3$H]Ins(4,5)$P_2$.

### Studies on the possible origins of [$^3$H]Ins$P_3$s identified in acid extracts of [$^3$H]Ins-prelabelled avian erythrocytes

When 0.1–0.5 $\mu$M-[$^3$H]Ins(3,4,5,6)$P_4$ was incubated with avian erythrocyte lysates [containing approx. 4 $\mu$M endogenous Ins(3,4,5,6)$P_4$], the rate of dephosphorylation, in either the presence or absence of ATP, was approx. 1 % of its rate of phosphorylation in the presence of ATP (results not shown).

The [$^3$H]Ins$P_3$ produced by the dephosphorylation reaction yielded [$^3$H]xylitol upon periodate oxidation, reduction and dephosphorylation, consistent with it having the structure [$^3$H]Ins(3,4,5)$P_3$. Human erythrocyte ghosts and a 40 kDa protein in rat brain cytosol also removed the 6-phosphate from [$^3$H]Ins(3,4,5,6)$P_4$ (results not shown).

Taken together these results suggest that the major [$^3$H]Ins$P_3$ present in peak III, that yielded [$^3$H]xylitol upon periodate oxidation, reduction and dephosphorylation (see above) and would hence be either [$^3$H]-

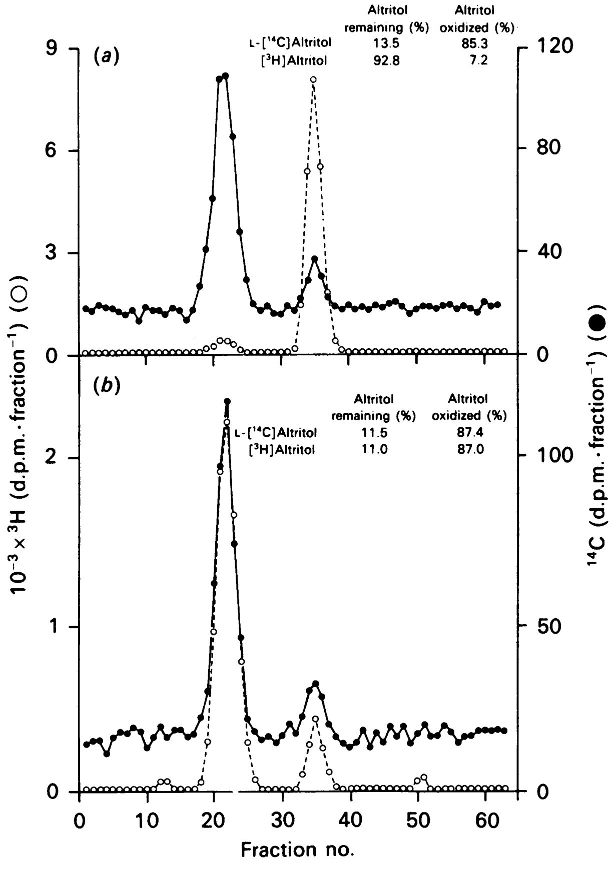

**Fig. 11. Characterization of the products of $[^3H]Ins(1,3,4,6)P_4$ phosphomonoesterase activities partially purified from rat brain cytosol**

$[^3H]Ins(1,3,4,6)P_4$ was incubated with aliquots of either the 150 kDa or the 31 kDa rat brain cytosol $[^3H]Ins(1,3,4,6)P_4$ phosphomonoesterase activities resolved from rat brain cytosol by gel-permeation chromatography (see the legend to Fig. 10 and the Materials and methods section for details of the assay conditions). The $[^3H]InsP_3$ products were resolved by anion-exchange h.p.l.c. (on a Partisil 10-SAX, h.p.l.c. column eluted as described in Stephens *et al.*, 1988*a*; results not shown). The assays containing aliquots of the 150 kDa proteins produced a single peak of $[^3H]InsP_3$(s) which eluted close to [$^{32}$P]ATP and yielded [$^3$H]altritol upon periodate oxidation, reduction and dephosphorylation (85 % of the $^3$H originally contained in the $[^3H]InsP_3$ was recovered in [$^3$H]altritol; results not shown). The assays containing the 31 kDa proteins were treated in an identical manner; two $[^3H]InsP_3$ isomers were resolved. The earlier eluting isomer (constituting between 70–80 % of the total $[^3H]InsP_3$s produced by these activities in three different experiments) eluted close to [$^{32}$P]ATP and yielded [$^3$H]altritol upon periodate oxidation, reduction and dephosphorylation (with an overall recovery of 84 %; results not shown). The peak with the longer retention time yielded [$^3$H]iditol upon periodate oxidation, reduction and dephosphorylation (with an overall recovery of 49 %) and was completely resistant to oxidation by L-iditol dehydrogenase (results not shown). The [$^3$H]altritols derived from the 150 kDa activity (*b*) or 31 kDa activity (*a*) were mixed with 20 $\mu$g of L-altritol and L-[$^{14}$C]altritol and incubated with 9.5 units of dehydrogenase/ml (see the Materials and methods section for details). The reaction was terminated, desalted and the products were resolved on a cation-exchange h.p.l.c. column (in the $Pb^{2+}$ mode, as described). The proportions of [$^{14}$C]- and [$^3$H]altritol oxidized were estimated by counting individual fractions of column eluate for $^{14}$C and $^3$H radioactivity, utilizing standard dual-label liquid scintillation counting techniques.

$Ins(3,4,5)P_3$ or $[^3H]Ins(1,5,6)P_3$ (see Table 1) is probably, in fact, $[^3H]Ins(3,4,5)P_3$.

$[^3H]Ins(1,3,4,6)P_3$ (0.1–0.2 $\mu$M) was dephosphorylated by avian erythrocyte lysates [the lysates contained approx. 0.5 $\mu$M endogenous $Ins(1,3,4,6)P_4$] in the presence of 5 mM-MgATP at approximately the same rate as it was phosphorylated. The $[^3H]InsP_3$s, which were produced during the incubation of $[^3H]Ins(1,3,4,6)P_4$ with chick erythrocyte lysates, could be resolved into two peaks by anion-exchange h.p.l.c. (results not shown). The earlier eluting $[^3H]InsP_3$(s) migrated close to [$^{32}$P]ATP and the later one(s) close to $Ins[^{32}P](1,4,5)P_3$. When these two $^3$H peaks were desalted, oxidized with sodium periodate, reduced and dephosphorylated, the first elution yielded [$^3$H]altritol {81 % recovery of $^3$H radioactivity in the original $[^3H]InsP_3$(s)}. Incubation with L-iditol dehydrogenase in the presence of L-[$^{14}$C]altritol showed that the [$^3$H]altritol was 100 ± 2 % L-altritol. The $[^3H]InsP_3$ with the longer retention time, resulting from the dephosphorylation of $[^3H]Ins(1,3,4,6)P_4$, yielded [$^3$H]iditol (45 % recovery of the radioactivity in the initial $[^3H]InsP_3$) which was 65.6 % L-[$^3$H]iditol and 34.4 % D-[$^3$H]iditol. This suggests that, although $[^3H]Ins(1,3,4)P_3$ and $[^3H]Ins(3,4,6)P_3$ are the major dephosphorylation products of $[^3H]Ins(1,3,4,6)P_4$ by avian erythrocyte lysates, a significant quantity of $[^3H]Ins(1,4,6)P_3$ is also formed.

Rat brain cytosol contained enzymes capable of dephosphorylating $[^3H]Ins(1,3,4,6)P_4$ by similar routes to those described above for avian erythrocyte cytosol (results not shown). Gel-filtration chromatography allowed some of the enzymes catalysing these reactions to be separated (see Fig. 10), although no $[^3H]Ins(1;3,4,6)P_4$ 1-phosphate phosphomonoesterase activity could be detected in the column eluate. An activity with a native molecular mass of 150 kDa dephosphorylated $[^3H]Ins(1,3,4,6)P_4$ to a $[^3H]InsP_3$ that gave L-[$^3$H]altritol upon periodate oxidation, reduction and dephosphorylation. 96 % of the $[^3H]InsP_3$ radioactivity was recovered in [$^3$H]altritol, of which 87.0 % was oxidized during an incubation with L-iditol dehydrogenase in which 87.4 % of an internal 'spike' of L-[$^{14}$C]altritol was oxidized (see Fig. 11), consistent with the structure $[^3H]Ins(1,3,4)P_3$.

Two additional $[^3H]Ins(1,3,4,6)P_4$ phosphomonoesterase activities co-eluted from the gel filtration column. One generated a $[^3H]InsP_3$ which could be separated from the product of the second activity by anion-exchange h.p.l.c. (on a Partisil 10 SAX column, eluted as described). The product of this activity yielded D-[$^3$H]altritol upon periodate oxidation, reduction and dephosphorylation (85 % of the original $[^3H]InsP_3$(s) was recovered as [$^3$H]altritol of which a minimum of 91.5 % was L-[$^3$H]altritol; see Fig. 11) suggesting it had the structure $[^3H]Ins(1,3,6)P_3$. The second activity produced an $[^3H]InsP_3$, which when purified by anion-exchange h.p.l.c. and desalted, yielded D-[$^3$H]iditol upon periodate oxidation, reduction and dephosphorylation (40 % of the radioactivity in the original $[^3H]InsP_3$ was recovered in [$^3$H]iditol, of which less than 1% was oxidized by L-iditol dehydrogenase during an assay in which 98.5% of an internal 'spike' of L-iditol was oxidized to sorbose) suggesting that the product of this enzyme was [$^3$H]Ins(1,4,6)$P_3$. Both the [$^3$H]Ins(1,3,4,6)$P_4$ 3-phosphate phosphomonoesterase and [$^3$H]Ins(1,3,4,6)$P_4$ 4-phosphate phosphomonoesterase activities eluted at volumes suggesting that they both possessed a native molecular mass of 31 kDa (see Fig. 10).

These data argue that three of the previously undefined [$^3$H]Ins$P_3$ isomers identified in acid extracts of avian erythrocytes, i.e. [$^3$H]Ins(1,3,6)$P_3$, [$^3$H]Ins(3,4,6)$P_3$ and [$^3$H]Ins(1,4,6)$P_3$, could be derived *in vivo* from the dephosphorylation of [$^3$H]Ins(1,3,4,6)$P_4$.

## DISCUSSION

The data presented in the Results section show that three peaks of [$^3$H]Ins$P_3$ isomers can be resolved from acid extracts of [$^3$H]Ins-prelabelled avian erythrocytes by anion-exchange h.p.l.c. The earliest eluting peak (designated I, see Fig. 3) contained 91% [$^3$H]Ins(1,3,4)$P_3$ and 9% [$^3$H]Ins(1,3,6)$P_3$. The analogue of this [$^3$H]Ins$P_3$ peak in carbachol-stimulated parotid slices was originally characterized as D- or L-Ins(1,3,4)$P_3$ by Irvine *et al.*, (1984). At the time of the original description of D- or L-Ins(1,3,4)$P_3$, there were no grounds for predicting which isomer(s) was present in the cell. With the later discovery of D- or L-Ins(1,3,4,5)$P_4$ in cell extracts (Batty *et al.*, 1985) and the characterization, in the test tube, of widely distributed enzymes capable of phosphorylating D-Ins(1,4,5)$P_3$ on the 3-OH and dephosphorylating the resulting D-Ins(1,3,4,5)$P_4$ at the 5-position to yield D-Ins(1,3,4)$P_3$, it became generally accepted that the cellular Ins$P_3$ was very likely to be D-Ins(1,3,4)$P_3$ (Irvine *et al.*, 1986; Hawkins *et al.*, 1986). The above set of assumptions has proved to be essentially correct for avian erythrocytes, although two unexpected features have emerged. First, [$^3$H]Ins(1,3,4)$P_3$ can also be derived, at least in rat brain cytosol and avian erythrocyte lysates, from [$^3$H]Ins(1,3,4,6)$P_4$ and second, a relatively small amount of [$^3$H]Ins(1,3,6)$P_3$ is clearly present in these cells. As a 150 kDa enzyme in rat brain cytosol can metabolize [$^3$H]Ins(1,3,4,6)$P_4$ to [$^3$H]Ins(1,3,6)$P_3$ (see above), cellular [$^3$H]Ins(1,3,6)$P_3$ might be derived from this source.

The second eluting [$^3$H]Ins$P_3$ peak, designated II (see Fig. 3), which eluted close to Ins[$^{32}$P](1,4,5)$P_3$, contained 72% [$^3$H]Ins(1,4,5)$P_3$, 20% [$^3$H]Ins(3,4,6)$P_3$ and 8% [$^3$H]Ins(1,4,6)$P_3$.

Since the original descriptions of PtdIns(4,5)$P_2$ phosphodiesterase activities in animal cells (Thompson & Dawson, 1964*a*,*b*,*c*), Ins(1,4,5)$P_3$ has been an expected constituent of the cytosol of most tissues. Irvine *et al.* (1984) showed that D- or L-[$^3$H]Ins(1,4,5)$P_3$ and/or D- or L-[$^3$H]Ins(1,4,6)$P_3$ is (are) present in acid extracts of carbachol-stimulated parotid gland slices.

The data above show that acid extracts of unstimulated avian erythrocytes contain three of these four alternative isomers, although the major species is indeed the expected product of phospholipase C-catalysed cleavage of PtdIns(4,5)$P_2$, i.e. Ins(1,4,5)$P_3$. The metabolic origin of the three minor species of [$^3$H]Ins$P_3$ found in peak II remains to be proven, but as [$^3$H]Ins-prelabelled avian erythrocytes only contain Ptd[$^3$H]Ins(4,5)$P_2$, and moreover [$^3$H]Ins(1,3,6)$P_3$, [$^3$H]Ins(3,4,6)$P_3$ and [$^3$H]Ins(1,4,6)$P_3$ can be generated from [$^3$H]Ins(1,3,4,6)$P_4$ in avian erythrocyte lysates and/or rat brain cytosol fractions, it is possible that these phosphomonoesterase activities might be responsible for their synthesis *in vivo*.

The most polar of the [$^3$H]Ins$P_3$ peaks isolated from acid extracts of [$^3$H]Ins-prelabelled avian erythrocytes (peak III) possesses properties consistent with the structures D- or L-[$^3$H]Ins(1,5,6)$P_3$ (see Table 1). The ability of avian erythrocyte lysates and a 45 kDa protein in rat brain cytosol to produce [$^3$H]Ins(3,4,5)$P_3$ from [$^3$H]Ins(3,4,5,6)$P_4$ suggests [by the application of arguments analogous to those applied to the identification of Ins(1,3,4)$P_3$ in cells (Irvine *et al.*, 1986)] that the material detected in cells is likely to be [$^3$H]Ins(3,4,5)$P_3$.

Several potentially significant [$^3$H]inositol trisphosphates could not be detected in significant quantities in acid extracts of avian erythrocytes. Although the phosphodiester bond in Ins(1:2-cyclic,4,5)$P_3$ is acid-labile, one of the products of its hydrolysis, Ins(2,4,5)$P_3$ [typically obtained in 10–20% yield from acid-treated Ins(1:2-cyclic,4,5)$P_3$; Hawkins *et al.*, 1987], should yield [$^3$H]glucitol upon periodate oxidation, reduction and dephosphorylation. In acid extracts from several independent preparations of [$^3$H]Ins-prelabelled avian erythrocytes, less than 1% of the $^3$H recovered in the polyols derived by periodate oxidation, reduction and dephosphorylation of a total [$^3$H]Ins$P_3$ fraction was in [$^3$H]glucitol. Because of the relatively low sensitivity of this technique (a consequence of the fact that the maximum yield of [$^3$H]glucitol from [$^3$H]Ins(1:2-cyclic,4,5)$P_3$ is 10–20%), this strategy certainly cannot rule out the presence of Ins(1:2-cyclic,4,5)$P_3$ in avian erythrocytes. However, a similar analysis of unstimulated human 1321 N1 astrocytoma cells showed that [$^3$H]glucitol made up 20% of the [$^3$H]polyols recovered after periodate oxidation, reduction and dephosphorylation of a total [$^3$H]Ins$P_3$ fraction (results not shown), indicating that [$^3$H]Ins(1:2-cyclic,4,5)$P_3$ might well make up a substantial proportion of the total [$^3$H]Ins$P_3$s in unstimulated 1321 N1 cells. Clearly this suggests that there is a significant amount of variation in the relative levels of these different inositol phosphates between one cell type and another.

A large number of studies have attempted to measure changes in Ins(1,4,5)$P_3$ concentration by labelling cells or tissues slices with [$^3$H]Ins and then quantifying the radioactivity eluted in an anion-exchange h.p.l.c.-purified [$^3$H]Ins(1,4,5)$P_3$ peak and assuming this value is directly proportional to Ins(1,4,5)$P_3$ concentration. In situations where the [$^3$H]Ins tracer has been introduced into the cell or tissue system relatively briefly, this assumption is probably correct {within the errors imposed by changes in the specific radioactivity of Ptd[$^3$H]Ins(4,5)$P_2$}. Only in situations where substantial quantities of [$^3$H]Ins-labelled metabolites have begun to accumulate in 'control' [$^3$H]Ins$P_3$, [$^3$H]Ins$P_4$ and [$^3$H]Ins$P_5$ fractions (this usually occurs after more prolonged periods, e.g. 6 h of labelling with [$^3$H]Ins, and is quite variable between different lines of cultured cells: L. Stephens, unpublished work) and where changes in '[$^3$H]Ins(1,4,5)$P_3$' are relatively small, e.g. after brief periods of agonist-induced stimulation, are errors in estimates of Ins(1,4,5)$P_3$ concentration introduced by 'background' [$^3$H]Ins$P_3$s likely to become substantial. The assumption that 'background' radioactivity in '[$^3$H]Ins(1,4,5)$P_3$ fractions' is universally explained by large quantities of other [$^3$H]Ins$P_3$s is possibly inaccurate, as most cell types contain relatively less Ins(1,3,4,5,6)$P_5$ than avian erythrocytes and might, therefore, be expected to contain relatively lower concentrations of the intermediates involved in its metabolism.

The presence of [$^3$H]Ins(3,4,5,6)$P_4$ in both acid and neutral extracts of a number of types of cells has been reported (Stephens *et al.*, 1988*a,c*). If an inositol trisphosphate serves as a direct precursor of this molecule, only four possible Ins$P_3$ isomers could be responsible: Ins(3,4,6)$P_3$, Ins(3,4,5)$P_3$, Ins(4,5,6)$P_3$ or Ins(3,5,6)$P_3$ (see Table 1). As none of the [$^3$H]Ins$P_3$s in an acid extract of [$^3$H]Ins-prelabelled avian erythrocytes co-eluted with Ins[$^{32}$P](4,5,6)$P_3$ from a WAX anion-exchange h.p.l.c. column, this isomer can be eliminated as a likely precursor. It should be noted that if cells are labelled with *myo*-[2-$^3$H]inositol then [$^3$H]Ins(4,5,6)$P_3$ cannot be detected and/or analysed by conventional periodate oxidation techniques as its C-2 is lost during periodate oxidation. Similarly, no [$^3$H]Ins(3,5,6)$P_3$ could be detected in acid extracts of [$^3$H]Ins-labelled avian erythrocytes indicating that this species is unlikely to be the precursor of Ins(3,4,5,6)$P_4$. The two remaining Ins$P_3$s that are potential precursors of Ins(3,4,5,6)$P_4$ have both been found in acid extracts of avian erythrocytes. The fact that Ins(3,4,5)$P_3$ is the product of a phosphomonoesterase activity that is present in several tissue types suggests that it might represent an intermediate in the degradative half of the pathway(s) metabolizing Ins(3,4,5,6)$P_4$; as such it is unlikely to simultaneously act as a precursor of Ins(3,4,5,6)$P_4$ [although the metabolic relationship between PtdIns4$P$ and PtdIns(4,5)$P_2$ is an example of just such a situation]. Thus Ins(3,4,6)$P_3$ is arrived at as the most likely Ins$P_3$ precursor of Ins(3,4,5,6)$P_4$ by a rather weak process of elimination on the grounds of relative cellular concentrations and the substrate specificity of the inositol phosphate phosphomonoesterase activities that can be detected in a number of tissues.

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