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L-*myo*-Inositol 1,4,5,6-tetrakisphosphate is present in both mammalian and avian cells

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When *myo*-[^{3}H]inositol-prelabelled primary-cultured murine bone-marrow-derived macrophages were challenged with platelet-activating factor (PAF; 200 ng/ml), there was a rapid (2.5-fold at 10 s) rise in the intracellular concentration of D-*myo*-[^{3}H]inositol 1,4,5-trisphosphate, followed by a rise in *myo*-[^{3}H]inositol tetrakisphosphate. *myo*-[^{3}H]Inositol tetrakisphosphate fractions were isolated by high-performance anion-exchange chromatography from *myo*-[^{3}H]inositol-prelabelled chick erythrocytes and primary-cultured macrophages. In both cases [^{3}H]iditol and [^{3}H]inositol were the only significant products (> 90% of recovered radioactivity) after oxidation to completion with periodic acid, reduction with $NaBH_4$ and dephosphorylation with alkaline phosphatase. The presence of [^{3}H]inositol after this procedure is consistent with the occurence of [^{3}H]inositol 1,3,4,5-tetrakisphosphate in the cell extracts, whereas [^{3}H]iditol could only be derived from D- or L-inositol 1,4,5,6-tetrakisphosphate. When [^{3}H]inositol tetrakisphosphate fractions obtained from (A) unstimulated macrophages, (B) macrophages that had been stimulated with PAF for 40 s or (C) chick erythrocytes were subjected to the above procedure, radioactivity was recovered in these polyols in the following proportions: A, 60–90% in iditol, with 10–40% in inositol; B, total radioactivity increased by a factor of 9.8, 94% being recovered in inositol and 8% in iditol; C, 70–80% in iditol and 20–30% in inositol. [^{3}H]Iditol derived from *myo*-[^{3}H]inositol tetrakisphosphate fractions from macrophages and chick erythrocytes was oxidized to sorbose by L-iditol dehydrogenase (L-iditol:NAD^+ 2-oxidoreductase, 1.1.1.14) at the same rate as authentic L-iditol. D-[^{14}C]Iditol, derived from D-*myo*-inositol 1,4,5-trisphosphate, was not oxidized by L-iditol dehydrogenase. This result indicates that the [^{3}H]iditol was derived from L-*myo*-inositol 1,4,5,6-tetrakisphosphate. The data are consistent with rapid PAF-sensitive synthesis of D-*myo*-[^{3}H]inositol 1,3,4,5-tetrakisphosphate in macrophages, and demonstrate that L-*myo*-inositol 1,4,5,6-tetrakisphosphate is synthesized in both mammalian and avian cells. The levels of L-*myo*-[^{3}H]inositol 1,4,5,6-tetrakisphosphate in primary-cultured macrophages are not acutely sensitive to PAF.

INTRODUCTION

fMet-Leu-Phe and PAF activate primary-cultured mouse bone-marrow-derived macrophages just as a variety of agonists stimulate other cells: via a receptor-regulated inositol phospholipid-selective phospholipase C (Downes & Michell, 1985; Whetton *et al.*, 1986). The pulses of D-Ins(1,4,5)P_3 and 1,2-diacylglycerol generated by this enzyme act as second messengers, co-ordinating cellular reponses to these agonists; in the case of activated macrophages this includes synthesis of numerous eicosanoids, secretion of PAF and generation of superoxide ions (North, 1981).

Fairly recently the hormone-stimulated production of D-Ins(1,3,4,5)P_4 has been described (Batty *et al.*, 1985). This molecule is synthesized by D-Ins(1,4,5)P_3 kinase (Irvine *et al.*, 1986) and may be implicated in the regulation of the processes which allow cytosolic calcium to be used as a transducing signal by allowing the entry of extracellular calcium into the cell interior under certain circumstances (Irvine & Moor, 1986).

While studying the role of D-[^{3}H]Ins(1,3,4,5)P_4 in the response of primary cultured macrophages to PAF, we found certain anomalies in the chromotography of InsP_4 fractions. Near-perfect co-chromatography was observed between authentic D-Ins[^{32}P](1,3,4,5)P_4 and [^{3}H]Ins-labelled InsP_4 derived from PAF-stimulated macrophages. However, [^{3}H]Ins-labelled InsP_4 derived from unstimulated macrophages did not co-chromatograph with authentic D-Ins[^{32}P](1,3,4,5)P_4.

The experiments described here were aimed at delineating the reason for this difference. They show that both mammalian and avian cells contain L-*myo*-inositol 1,4,5,6-tetrakisphosphate.

Abbreviations used: Ins, *myo*-inositol; PtdIns, D-phosphatidyl-*myo*-inositol; PtdIns4*P*, D-phosphatidyl-*myo*-inositol 4-phosphate; PtdIns(4,5)P_2, D-phosphatidyl-*myo*-inositol 4,5-bisphosphate; D-Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; D-Ins(1,3,4,5)P_4, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; L-Ins(1,4,5,6)P_4, L-*myo*-inositol 1,4,5,6-tetrakisphosphate; Gro*P*Ins(4,5)P_2, glycerophosphoinositol 4,5-bisphosphate; PAF, platelet-activating factor; InsP_5, inositol pentakisphosphate; FMLCM, Fischer's medium for leukaemic cells of mice; fMet, formylmethionyl; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium.

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MATERIALS AND METHODS

Preparation of macrophages

Mouse bone-marrow-derived macrophages were cultured as described previously (Whetton *et al.*, 1986) with a number of significant exceptions. (1) The cells were seeded at a density of 0.8×10^6 cells/ml in a solution of 20% (v/v) horse serum (heat-inactivated; Gibco), 10% (v/v) L-cell conditioned medium (Whetton *et al.*, 1986), 1% antibiotics (streptomycin and penicillin, both at a final concentration of 50 i.u./ml), 69% (v/v) Fischer's medium for leukaemic cells of mice (FMLCM). (2) This medium was replaced with fresh after 4 days of culture. (3) After a further 2–3 days culturing, the monolayers of cells were rinsed once with FMLCM before recommencing incubation in a solution of FMLCM, 1% BSA (recrystallized fraction V; Sigma) and *myo*-[^{3}H]inositol (10–200 μCi/ml final concn.; New England Nuclear). After 20–21 h in this solution at 37 °C in a CO_2/air (1:19) atmosphere, the cells were prepared for experimental manipulation by removing the labelling medium and replacing it with an equal volume of FMLCM without $NaHCO_3$ (Gibco), containing 18 mM-sodium Hepes, pH 7.05 at 37 °C. This exercise was performed under sterile conditions, and it was only once the cells had stabilized at 37 °C in a water bath in the laboratory and the addition of experimental agents had commenced that sterile techniques were relaxed.

Preparation of acid extracts from macrophages

Cell incubations were terminated by the addition of an equal volume of ice-cold 10% (v/v) $HClO_4$ containing 250 μg of phytic acid/ml. The trays of cells were placed on ice and scraped clear with a silicone wedge. The final slurry of cell debris was quantitatively transferred to a 5 ml polypropylene test tube (Sarsted), centrifuged for 5 min at the maximum setting of a refrigerated bench-top centrifuge (Beckman; model TJ-6), and the supernatant removed for immediate neutralization (see below).

Preparation of a phospholipid extract from macrophages

Lipid extractions were performed on the pellet of $HClO_4$- or trichloroacetic acid-precipitated cell lysate, essentially as described by Creba *et al.* (1983). The dry lipid films produced by this strategy were deacylated, and the resulting [^{3}H]glycerophosphoinositol phosphate esters were separated by anion-exchange chromatography as described by Downes *et al.* (1986).

Preparation and incubation of chick erythrocytes

In all, 50 5-day-old chicks were rendered unconscious with CO_2, decapitated and their blood collected (approx. 1 ml/chick) in one of two ways. (1) If the blood was to be used for the preparation of chick serum, then it was collected directly into an ice-cold tube, where it was allowed to clot. Clotted blood, pooled from a number of chicks, was centrifuged for 30 min at the maximum setting of a refrigerated bench-top centrifuge. The serum was removed and filtered (0.22 μm-pore-size Sterivex GS; Millipore Corp.) under sterile conditions. Excess serum above immediate requirements was stored at −20 °C. (2) Blood was collected into 20 ml of ice-cold DMEM (without $NaHCO_3$; buffered with 25 mM-Hepes; Gibco) containing 500 units of heparin. The blood from 15 chicks was collected in this way, pooled, and pelleted in a refrigerated bench-top centrifuge, and the supernatant and buffy coat were aspirated and discarded. This was repeated four times, using sterile DMEM to resuspend the erythrocytes each time. Finally a 1.5 ml portion of packed erythrocytes was removed under sterile conditions ard resuspended in 5 ml of 74% (v/v) chick serum (prepared as described above)/25% (v/v) DMEM/1% antibiotics (as described above)/*myo*-[^{3}H]inositol (1 mCi) in a sterile culturing flask (50 ml; Gibco).

Another 1.5 ml portion of erythrocytes was resuspended in a solution identical with that described above, except that it contained 2 mCi of [^{32}P]P_i (Amersham PBS 11; carrier-free). Both flasks were shaken gently at 40 °C in an atmosphere of CO_2/O_2 (1:19).

Preparation of an acid extract from chick erythrocytes

After incubating for 35 h, the cells were transferred into 20 ml of ice-cold DMEM and pelleted once in a bench-top centrifuge. The medium was removed and the cells lysed by the addition of 5 ml of ice-cold water. After 4 min, 5 ml of ice-cold 10% $HClO_4$ was added and, after vigorous mixing, the precipitated cell material was centrifuged for 15 min at the maximum setting of a refrigerated bench-top centrifuge. This protocol was adopted as it yielded a more manageable precipitate than that which resulted from the direct addition of $HClO_4$ to the cell pellet. The supernatant was removed for neutralization as described below.

Neutralization and extraction of $HClO_4$ in tissue extracts

Tri-n-octylamine/Freon (1:1, v/v; Sharpes & McCarl, 1982) was added to the $HClO_4$ cell extracts. After very vigorous mixing (> 15 s), the samples were centrifuged (10 min at the maximum setting of a refrigerated bench-top centrifuge). The upper phases were taken and chromatographed directly after the addition of 50 μl of 0.1 M-EDTA, pH 7.0. If samples were to be subjected to h.p.l.c., then they were filtered (0.45 μm-pore-size filters; GS).

High-performance anion-exchange chromatography of inositol phosphates

H.p.l.c. was performed using a Partisil 10-SAX column [25 cm × 0.46 cm (main column) and 2 cm × 0.46 cm (guard column); Jones Chromatography, Hengoed, Mid-Glamorgan, Wales, U.K.]. The gradient used was delivered by two independent pumps from buffer reservoirs: A, containing water, and B, containing 3.5 M-ammonium formate/H_3PO_4, pH 3.7 at 22 °C, at a flow rate of 1.25 ml/min. The following gradient was used (%B): 0 min, 0%; 5 min, 0%; 10 min, 21.4%; 12 min, 21.4%; 18 min, 28.5%; 23 min, 28.6%; 30 min, 40%; 40 min, 42%; 60 min, 99.9%; 65 min, 99.9%; 67 min, 0%; 70 min, 0%. Under these conditions the system had a void time to the fraction collector of 7.5 min. All Figures displaying gradients are corrected accordingly.

Desalting radiolabelled inositol phosphate fractions from h.p.l.c. gradients

The method of Downes *et al.* (1986) was employed. Essentially the fractions containing the [^{3}H]InsP_4 were pooled and neutralized with triethylamine, then diluted 5-fold with water and applied to a column of Bio-Rad AG 1 (X8; formate form; 200–400 mesh) anion-exchange resin (200 μl packed volume). The resin was washed once with 1.5 ml of 0.5 M-NaOH and then with 2 × 2 ml of

2 M-NaOH; each time the resin was resuspended in the 2 M-NaOH and left for 10 min before being drained. Then 4 ml of 2 M-NaOH was applied to 10 ml of Bio-Rad AG 50W cation-exchange resin (X4; acid form; 200–400 mesh) that had been thoroughly washed first with 2 M-NaOH, then with 2 M-HCl and finally with water. The eluate was collected and the resin washed once with 10 ml of water. These washes were combined, freeze-dried and finally dissolved in 1 ml of water. The pH of this solution was adjusted to 6.9–7.1 by the addition of a small volume of 2 M-KOH/0.2 M-Hepes. Recoveries through this process were in the range of 75–95%.

Preparation of standards

Inositol phosphates. D-Ins[^{32}P](1,4,5)P_3 was obtained from [^{32}P]P_i-labelled human erythrocytes, by Ca^{2+}-activation of the membrane-associated, phosphoinositidase C (Downes & Michell, 1981). D-[^{3}H]Ins(1,3,4,5)P_4 was prepared as follows: D-[^{3}H]Ins(1,4,5)P_3 (50000 d.p.m./pmol; Amersham International) was incubated with 5.0 mM-ATP, 70 mM-potassium glutamate, 10 mM-Hepes, 30 mM-NaCl, pH 7.2, and a sufficient quantity of a partially purified preparation of rat brain cytosol D-Ins(1,4,5)P_3 kinase (Morris *et al.*, 1988) to ensure complete conversion into [^{3}H]InsP_4. D-Ins[^{32}P]-(1,3,4,5)P_4 was prepared in a similar manner, but using D-Ins[^{32}P](1,4,5)P_3 as the substrate.

D-[^{14}C]Ins(1,4,5)P_3 was prepared by incubating primary-cultured macrophages with [^{14}C]Ins (20 μCi/ml; Amersham International) in a 3-cm-diameter culturing dish for 24 h (see above). A phospholipid extract was prepared as described above and deacylated with methylamine (Clarke & Dawson, 1981). The [^{14}C]-glycerophosphoinositol phosphates produced were oxidized (in the dark) with periodate (0.5 ml of 10 mM-sodium periodate, pH 5.5, for 30 min; Brown & Stewart, 1966). The remaining oxidizing reagent was removed with ethylene glycol (a 5-fold molar excess with respect to periodate; the reaction was allowed to go to completion over 30 min incubation at 25 °C in the dark). The aldehyde groups remaining on the phosphodiester phosphates were eliminated by reaction with an aq. 1% 1:1-dimethylhydrazine (adjusted to pH 4.5 with formic acid; the reaction was allowed to proceed for 4 h at 25 °C). The mixture of [^{14}C]inositol phosphates produced by this procedure was passed through 5 ml of Bio-Rad AG-50W cation-exchange resin (X4; 200–400 mesh; acid form), neutralized, freeze-dried, and applied to an h.p.l.c. column (Partisil 10-SAX) in 1 ml of 10 mM-EDTA, pH 7.0. The fractions containing D-[^{14}C]Ins(1,4,5)P_3 (12000 d.p.m., and amounting to 6% of the total [^{14}C]-inositol phosphate preparation) were pooled, neutralized and desalted (as described above), yielding a sample of radiochemically pure D-[^{14}C]Ins(1,4,5)P_3. The authenticity and specific radioactivity of the preparation was assessed by incubating a portion of the [^{14}C]Ins(1,4,5)P_3 with a partially purified preparation of rat brain cytosol D-Ins(1,4,5)P_3 kinase (Morris *et al.*, 1988) in the presence of [γ^{32}P]ATP of known specific radioactivity, and under conditions leading to the complete conversion of the available substrate into D-[^{14}C]Ins[1-^{32}P](1,3,4,5)P_4. The quantity of ^{32}P incorporated was measured and the concentration, and hence the specific radioactivity, of the substrate calculated directly (results now shown). The specific radioactivity of the D-[^{14}C]Ins(1,4,5)P_3 was about 200 d.p.m./pmol.

D-[^{3}H]Ins(1,3,4)P_3 was prepared by 5-phosphate-specific dephosphorylation of D-[^{3}H]Ins(1,3,4,5)P_4, using the InsP_4 5-phosphatase present in human erythrocyte membranes.

Each inositol phosphate was finally purified by h.p.l.c. on a Partisil 10-SAX column and desalted as described above.

Polyols. D-Glucitol (sorbitol) and L-sorbose were purchased from Aldrich. *myo*-Inositol was purchased from BDH. D-[^{14}C]Glucitol (sorbitol) and *myo*-[^{14}C]-inositol were purchased from Amersham International.

D-Altritol, L-iditol and D-iditol were prepared from D-altrose, L-idose and D-idose (all purchased from Sigma) respectively by reduction with $NaBH_4$ (Grado & Ballou, 1961). A sample (10 mg) of the appropriate sugar was dissolved in 1.0 M-$NaBH_4$ (0.5 ml) and the resulting solution left in an open-topped vessel at 25 °C for 10–12 h. The excess $NaBH_4$ was removed as described below. Some of the experiments presented here used L-iditol given to us by Dr. N. Baggett (Department of Chemistry, University of Birmingham, Birmingham, U.K.).

Production of [^{3}H]- and [^{14}C]-polyols from [^{3}H]- and [^{14}C]-inositol phosphates

The strategy used was periodate oxidation of the inositol phosphate of interest followed by reduction and dephosphorylation to yield a polyol. The methods used were based upon those previously described by Grado & Ballou (1961), Johnson & Tate (1969) and Irvine *et al.* (1984).

A dried sample of the [^{3}H]- or [^{14}C]-inositol phosphate was dissolved in 0.1 M-periodic acid (pH adjusted to 2.0 with NaOH; 0.5 ml). The sample was then left in a closed vessel in the dark at 25 °C for 36 h. The aldehydes resulting from periodate oxidation, together with excess periodate, were reduced by addition of 1 M-$NaBH_4$ (0.5 ml) and leaving the sample in a vessel open to the air for a further 10–12 h at 25 °C. At this point, small amounts of the appropriate polyols (see below) were added to act as carriers for the ^{3}H-labelled material; these were added as a single portion (5 μl) containing 20–25 μg each of inositol, D-glucitol, D-altritol and L-iditol and approx. 2×10^3 d.p.m. of both *myo*-[^{14}C]-inositol and D-[^{14}C]glucitol. The borohydride was removed by acidification and conversion of the resulting boric acid into the volatile trimethylborane. This was achieved by first passing the sample through a small column (3 ml) of Bio-Rad AG-50W resin (X4; 200–400 mesh; H^+ form). The eluate was then freeze-dried, resuspended in methanol (10 ml) and freeze-dried again.

Dephosphorylation was achieved by using alkaline phosphatase under conditions previously designed to lead to the complete dephosphorylation of D-Ins[^{32}P]-(1,4,5)P_3 and D-Ins[^{32}P](1,3,4,5)P_4 (results not shown). The dried sample was dissolved in 10 mM-ethanolamine (pH 9.5)/1 mM-$MgSO_4$, containing 20 units of bovine intestinal alkaline phosphatase (type P-5521, Sigma; units defined in glycine buffer)/ml in a final volume of 2 ml, and left at 25 °C for 10–12 h. Finally the sample was desalted by passing it through a column of mixed-bed ion-exchange resin (Amberlite MB-3, Sigma; 2 ml). The sample was freeze-dried and then redissolved in a small volume of water. The polyols present in this sample

were resolved and quantified by h.p.l.c. with a column of cation-exchange resin in either the Ca^{2+} or Pb^{2+} form (see below).

Experience revealed that when [^{3}H]inositol phosphates were purified from Partisil 10-SAX h.p.l.c. eluate (as described above), a maximum of 25% of the total desalted preparation could be effectively oxidized by the quantity of periodic acid defined above. This is presumably a consequence of undefined periodate-sensitive material being carried through these different steps.

Separation of polyols was achieved with a Brownlee cation-exchange h.p.l.c. column (22 cm × 0.46 cm; Polypore-Ca; purchased from Anachem, Luton, Beds., U.K.) and a mobile phase of deionized water. A flow rate of 0.2 ml/min and a column temperature of 85–90 °C gave a good separation of inositol, altritol, glucitol and iditol (see Fig. 3 below), though, as might be expected, it could not distinguish between the two enantiomers of the sugar alcohols (results not shown). Detection of 5–20 μg of each polyol was achieved by monitoring changes in either refractive index (Waters 410 differential refractometer; sensitivity 4-8) or u.v. (200 nm) absorbance (Perkin–Elmer LC-95 instrument; 0.1 absorption units full scale), though the former method was preferred, owing to the relatively high u.v. absorbance of contaminants (present in low molar proportions) in the polyol preparations.

Radiolabelled polyols were detected by measuring the radioactivity in 0.5 min fractions of the column eluate. Neither measurement of refractive index nor u.v. absorbance could be used as an independent confirmation of the retention times of [^{3}H]polyols derived from [^{3}H]-inositol phosphates because the refractometer we used possessed a large post-cell dead volume which led to excessive ^{3}H-peak broadening, and the relatively large amounts of u.v.-absorbing material present in the samples masked out the u.v. absorbance attributable to some of the polyols. However, since [^{14}C]inositol and [^{14}C]-glucitol were included as internal standards for each run (see above), identification of ^{3}H-labelled material was unequivocal. Recoveries of radioactive polyols through this column were > 90%.

After 10–12 runs, a steady increase in column pressure and peak broadening was observed, and in later experiments a 3 cm × 0.46 cm replaceable guard column (containing the same resin) was used, leading to an increase in the retention times of the polyol standards (see the Results section).

During the course of this work we discovered that an equivalent h.p.l.c. column, with resin in the Pb^{2+} form (Brownlee; Polypore-Pb; 22 cm × 0.46 cm; Anachem), gave greater resolution of the polyols than the Ca^{2+}-form-resin column described above. This 'Pb^{2+}' column was used in a manner identical with that used for the 'Ca^{2+}' column in a small number of the later experiments (see Fig. 10 below).

Use of L-iditol dehydrogenase for the total structural assignment of the [^{3}H]iditol derived from [^{3}H]-InsP_4

L-Iditol dehydrogenase (polyol dehydrogenase; L-iditol:NAD^+ oxidoreductase: 1.1.1.14; Sigma) was used

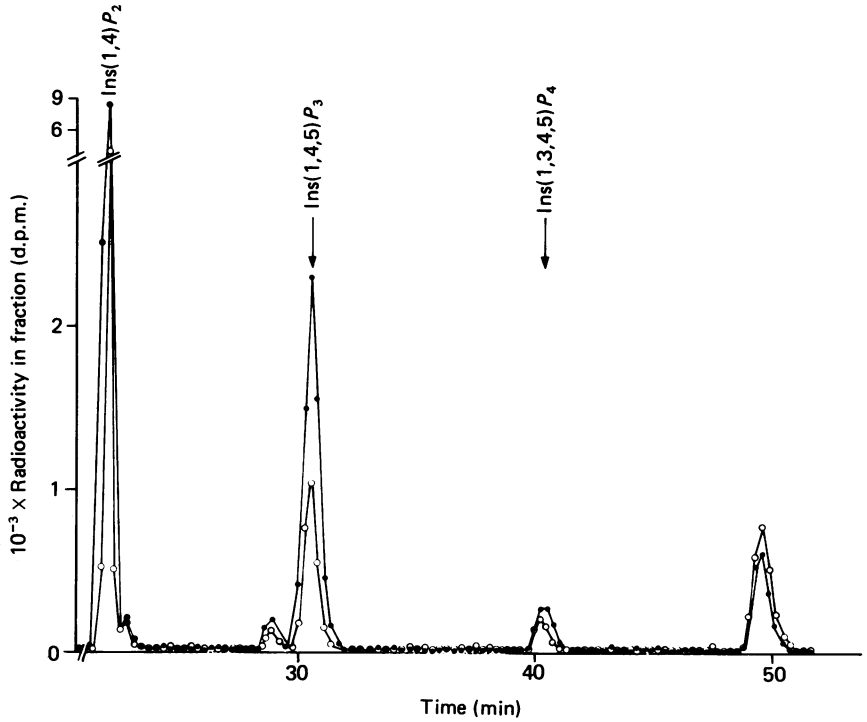

Fig. 1. H.p.l.c. profile of [^{3}H]Ins-labelled extracts prepared from macrophages

[^{3}H]Ins-prelabelled primary-cultured macrophages (20 μCi of [^{3}H]Ins/ml) were incubated in the presence of 200 ng of PAF/ml for 10 s (●) or vehicle (○). An acid extract of the cells was prepared, mixed with D-Ins[^{32}P](1,4,5)P_3 and D-Ins[^{32}P](1,3,4,5)P_4, and separated on a Partisil 10-SAX h.p.l.c. column (as described in the Materials and methods section). Fractions (0.25 min) were collected and counted for radioactivity. In this experiment a total (mean) of 59000 d.p.m. was applied to the h.p.l.c. column, of which 99% (mean) was recovered. The h.p.l.c. profiles displayed were representative of three replicates for each sample. The arrows indicate the positions of the peak fractions containing the ^{32}P-labelled standards.

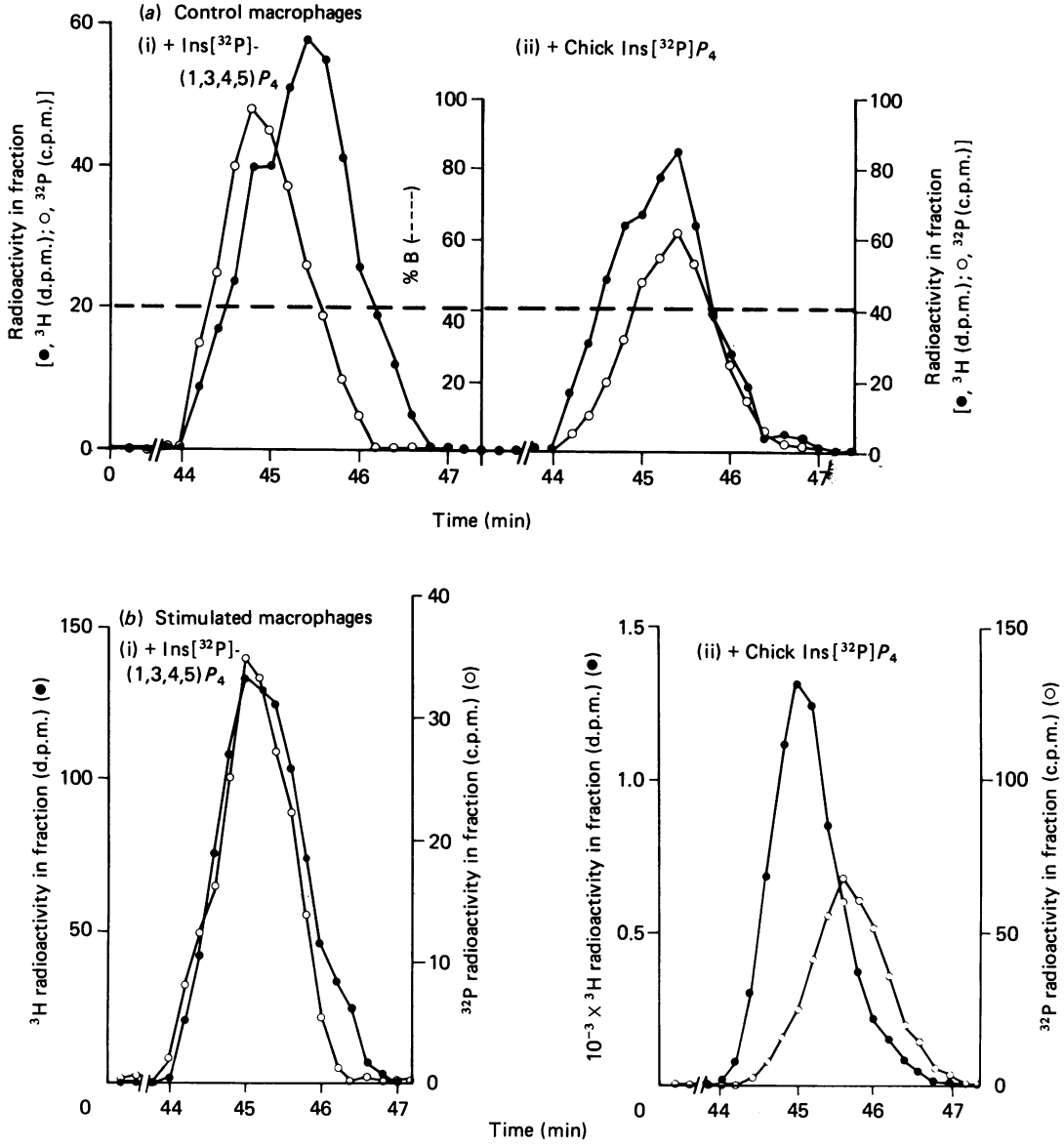

Fig. 2. Co-chromatography of [^{3}H]InsP_4 derived from macrophages with D-Ins[^{32}P](1,3,4,5)P_4 and Ins[^{32}P]P_4 from chicken erythrocytes

H.p.l.c.-purified desalted [^{3}H]InsP_4 from either stimulated or unstimulated macrophages (prelabelled with 200 μCi of [^{3}H]Ins/ml for 21 h) was separated on a Partisil 10-SAX h.p.l.c. column in the presence of ^{32}P-labelled standards. (*a*) Aliquots of a [^{3}H]InsP_4 fraction (11000 d.p.m. in total) from unstimulated macrophages were mixed with either (i) D-Ins[^{32}P](1,3,4,5)P_4 (3.4% of the [^{3}H]InsP_4 preparation was applied, of which 92% was recovered) or (ii) Ins[^{32}P]P_4 from chicken erythrocytes (4.5% of the [^{3}H]InsP_4 preparation was applied, of which 85% was recovered). (*b*) Aliquots of a [^{3}H]InsP_4 fraction from macrophages stimulated for 40 s with PAF (2 μg/ml) (the total d.p.m. in this fraction per 10^6 cells was 9.8 times higher than in the control macrophages and contained 107000 d.p.m.) were mixed with either (i) D-Ins[^{32}P](1,3,4,5)P_4 (1.0% of the [^{3}H]InsP_4 preparation was used, of which 88% was recovered) or (ii) Ins[^{32}P]P_4 from chicken erythrocytes (6.4% of the total [^{3}H]InsP_4 preparation was applied, of which 94% was recovered).

as a stereoselective tool to establish the D/L isomerism of the [^{3}H]iditol produced by periodate oxidation, reduction and dephosphorylation of macrophage and avian erythrocyte [^{3}H]InsP_4.

To establish the identity of the [^{3}H]iditol derived from macrophage and avian [^{3}H]InsP_4 the [^{3}H]polyol was incubated with L-iditol dehydrogenase (1.5 units/ml) in 100 mM-Tris/HCl (pH 8.3, 22 °C) and 20 mM-β-NAD$^+$ as part of a substrate mixture containing 70–100 μM-L-iditol and 1000 d.p.m. of D-[^{14}C]iditol/ml. The radiolabelled iditols constituted less than 0.01% of the total iditol present. A 1 ml portion of the reaction mixture was placed in a cuvette and the progress of the reaction was followed by monitoring the absorbance at 340 nm. When 30–76% of the starting L-iditol had been oxidized (see below), the reaction mixture was placed in a boiling-water bath for 3 min. The denatured enzyme was centrifuged out of the suspension in a bench-top centrifuge, and the supernatant was applied directly to 2 ml of Amberlite MB-3 mixed-bed ion-exchange resin.

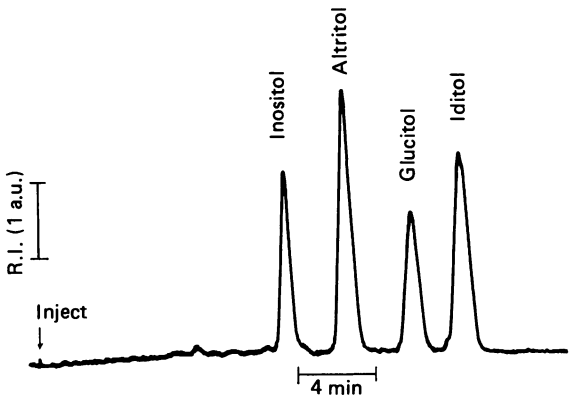

Fig. 3. H.p.l.c. separation of polyols

A mixture of *myo*-inositol, D-altritol, D-glucitol and L-iditol (approx. 25 μg of each, dissolved in 5 μl of water) was resolved by h.p.l.c. on a Brownlee 22 cm Polypore-Ca column, as described in the Materials and methods section. The flow rate was 0.2 ml/min. The polyols were detected by changes in the refractive index (R.I.) of the eluate. The peaks were identified by comparing their retention times with those of the pure polyols run in parallel. Abbreviation: a.u., arbitrary unit.

After being left for 30 min, the resin was drained and washed twice with 5 ml of water. The washings were pooled and freeze-dried. Recoveries of polyol through this procedure reproducibly exceeded 95%. The dried residue was resuspended in 10–25 μl of water and subjected to h.p.l.c. with a cation-exchange column in the Ca^{2+} form (as described above).

The extent of reaction in the sample was measured in terms of: (A) the [^{3}H]iditol (of unknown isomerism) converted into [^{3}H]sorbose; (B) the D-[^{14}C]iditol conversion into D-[^{14}C]sorbose; and (C) in terms of the quantity of L-iditol (present as a carrier) oxidized to L-sorbose. (A) and (B) were quantified by collecting the eluate from the h.p.l.c. column as fractions and counting them for ^{14}C and ^{3}H radioactivity; (C) was determined by spectrophotometric measurement of NADH production during the course of the reaction. The initial concentration of L-iditol in each sample was estimated by extrapolating the measured curves for NADH production to 100% reaction by best-fitting the data to an equation describing the progress of a first-order reaction (Draper & Smith, 1966); the percentage of the starting L-iditol that had been oxidized at the time the reactions were quenched was calculated from these data directly by assuming a 1:1 stoichiometry between L-iditol oxidation and NADH production (see below).

RESULTS

PAF-stimulated accumulation of inositol phosphates in macrophages

Exposure of [^{3}H]inositol-labelled macrophages to PAF (200 ng/ml) for 10 s provoked the rapid formation of ^{3}H-labelled compounds which co-chromatographed with D-Ins[^{32}P](1,4,5)P_3, D-Ins[^{32}P](1,4)P_2, D-Ins[^{32}P](1,3,4,5)P_4 and D-Ins[^{32}P](1,3,4)P_3 when analysed by strong-anion-exchange h.p.l.c. (Fig. 1; results not shown).

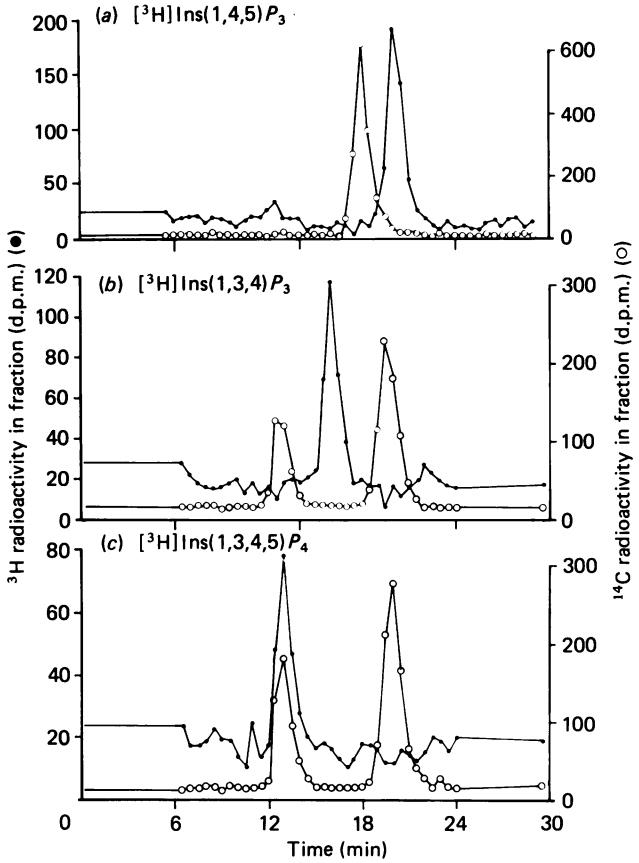

Fig. 4. Polyols derived from inositol phosphate standards

D-[^{3}H]Ins(1,4,5)P_3, D-[^{3}H]Ins(1,3,4)P_3 and D-[^{3}H]Ins-(1,3,4,5)P_4 were prepared, purified by h.p.l.c. on Partisil 10-SAX and desalted as described in the Materials and methods section. A sample of each *myo*-[^{3}H]inositol phosphate was oxidized with 0.1 M-periodic acid, pH 2.0, for 36 h at 25 °C then reduced by addition of $NaBH_4$. At this point an aliquot containing polyol standards, D-[^{14}C]-glucitol and *myo*-[^{14}C]inositol in the case of the samples derived from D-[^{3}H]Ins(1,3,4)P_3 and D-[^{3}H]Ins(1,3,4,5)P_4, and only D-[^{14}C]glucitol in the case of the sample derived from D-[^{3}H]Ins(1,4,5)P_3 (see the Materials and methods section) was added to each sample. Each sample was dephosphorylated by incubation with alkaline phosphatase. The [^{3}H]polyols produced were analysed by h.p.l.c. on a Brownlee Polypore-Ca^{2+} column. The flow rate was 0.2 ml/min. Fractions (0.5 min) of the column eluate were collected and their ^{14}C and ^{3}H radioactivities determined by dual-label liquid-scintillation counting. The proportions of starting ^{3}H and ^{14}C radioactivity recovered in the appropriate polyols were: [^{3}H]Ins(1,4,5)P_3: recovery of ^{3}H, 67%; recovery of [^{14}C]polyol, 75%; [^{3}H]Ins(1,3,4)P_3: recovery of ^{3}H, 71%; recovery of [^{14}C]polyols, 63%; [^{3}H]Ins(1,3,4,5)P_4: recovery of ^{3}H, 48%; recovery of [^{14}C]-polyols, 72%.

These results are consistent with previous work (Whetton *et al.*, 1986) showing that macrophages possess hormone receptors which couple to phosphoinositidase C. Activation of this enzyme leads to the rapid formation of D-Ins(1,4,5)P_3 and thus, via metabolism of this compound, to the formation of D-Ins(1,3,4,5)P_4 and D-Ins(1,3,4)P_3 (Batty *et al.*, 1985; Hawkins *et al.*, 1986; Irvine *et al.*, 1986).

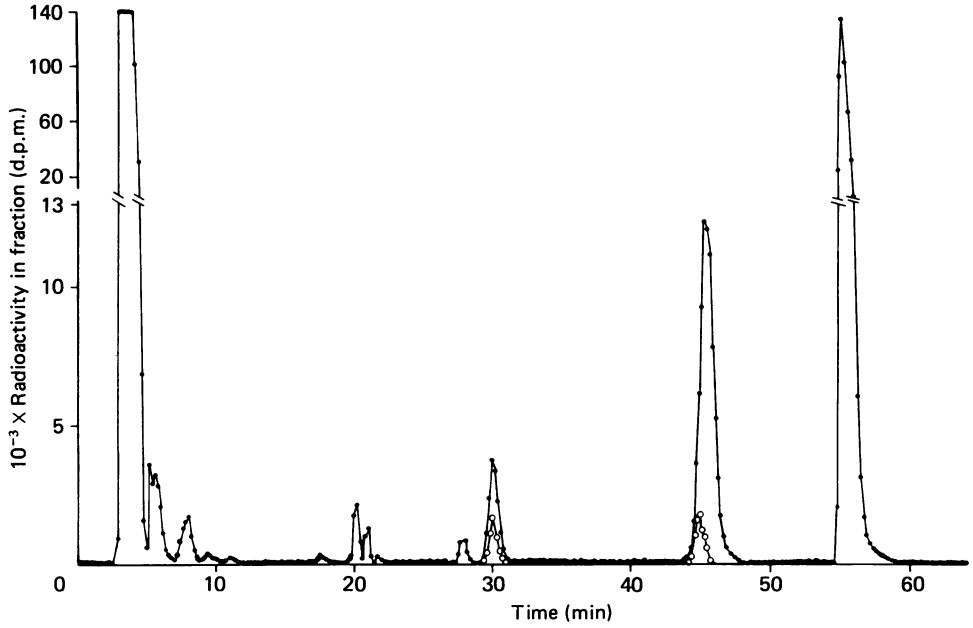

Fig. 5. H.p.l.c. profile of [^{3}H]Ins-labelled extract of chicken erythrocytes

Erythrocytes from 5-day-old chicks (1.5 ml) were labelled with [^{3}H]Ins (1 mCi in 5.5 ml total volume; see the Materials and methods section for details). After 35 h the cells were washed with ice-cold medium, lysed and extracted with $HClO_4$. A 150 μl portion of the neutralized acid extract was mixed with D-Ins[^{32}P](1,4,5)P_3 and D-Ins[^{32}P](1,3,4,5)P_4 and applied to a Partisil 10-SAX h.p.l.c. column, which was then eluted as described in the text. Fractions (0.2 min) were collected and counted for radioactivity. In all, 3.8×10^6 ^{3}H d.p.m. was applied, of which 101% was recovered: 85% was in [^{3}H]Ins, 2% in [^{3}H]InsP_4 and 12.5% in [^{3}H]InsP_5. ●, ^{3}H radioactivity; ○, ^{32}P radioactivity.

Detection of more than one inositol tetrakisphosphate in mouse bone macrophages

[^{3}H]Inositol-labelled macrophages were exposed to PAF (2 μg/ml) for 40 s, and the [^{3}H]inositol phosphates extracted and separated by h.p.l.c. The ^{3}H radioactivity which chromatographed in the InsP_4 region was then collected and desalted (see the Materials and methods section). The amount of [^{3}H]InsP_4 derived from stimulated cells was approx. 10-fold higher than that derived from control cells (see the legend to Fig. 2).

The two preparations of [^{3}H]InsP_4 were then re-subjected to h.p.l.c., but this time in the presence of two Ins[^{32}P]P_4 standards: D-Ins[^{32}P](1,3,4,5)P_4 prepared by phosphorylation of D-Ins[^{32}P](1,4,5)P_3 and Ins[^{32}P]P_4 isolated from chick erythrocytes labelled with [^{32}P]P_i (see the Materials and methods section). The major InsP_4 in chicken erythrocytes has been identified as either D- or L-Ins(1,4,5,6)P_4 (Johnson & Tate, 1969; see below).

As expected, [^{3}H]InsP_4 derived from stimulated cells co-chromatographed very closely with D-Ins[^{32}P]-(1,3,4,5)P_4, but not with chick Ins[^{32}P]P_4 (Fig. 2*b*), suggesting that this is the predominant isomer that is produced on stimulation. However, it was striking that [^{3}H]InsP_4 derived from unstimulated cells did not co-chromatograph with D-Ins[^{32}P](1,3,4,5)P_4 but chromatographed more closely with chick Ins[^{32}P]P_4 (see Fig. 2*a*). This result indicates that macrophages contain at least one isomer of InsP_4 that is distinct from D-Ins(1,3,4,5)P_4. Moreover, the relative retention times on h.p.l.c. of D-Ins[^{32}P](1,3,4,5)P_4, chick Ins[^{32}P]P_4 and [^{3}H]InsP_4 samples from control and stimulated macrophages suggests that unstimulated macrophages contain a mixture of InsP_4 species, the predominant component being chromatographically identical with an InsP_4 found in chick erythrocytes and the lesser component being D-Ins(1,3,4,5)P_4. Unfortunately the extent to which D-Ins[^{32}P](1,3,4,5)P_4 and chick Ins[^{32}P]P_4 were separated was not sufficient to allow a more quantitative analysis of the results.

Determination of the structure of InsP_4 species in mouse bone macrophages and avian erythrocytes

The problem of defining the intramolecular location of phosphate groups in radiochemical quantities of [^{3}H]-inositol phosphates has usually been tackled by utilizing the precisely defined structural requirements for cleavage of carbon–carbon bonds by periodic acid (Grado & Ballou, 1961). Only carbon–carbon bonds flanked by free hydroxy groups are potential sites for attack. Thus periodate oxidation of [^{3}H]Ins phosphates, followed by reduction and dephosphorylation, generates a polyol whose identity (and ^{3}H content) is determined by the site of free *vicinal* hydroxy moeities in the parent compound.

This method has been used by Irvine and his colleagues to identify some of the inositol phosphates which accumulate during cellular stimulation, namely D-Ins-(1,4,5)P_3 (Irvine *et al.*, 1984), D-Ins(1,3,4)P_3 (Irvine *et al.*, 1984) and D-Ins(1,3,4,5)P_4 (Batty *et al.*, 1985). The stereochemical configuration of these compounds was inferred from the earlier work of Grado & Ballou (1961) on the structure of D-PtdIns(4,5)P_2.

The above procedure was tested by applying it to three inositol phosphates of known structure. In each case the expected polyol was formed (Figs. 3 and 4); [^{3}H]iditol

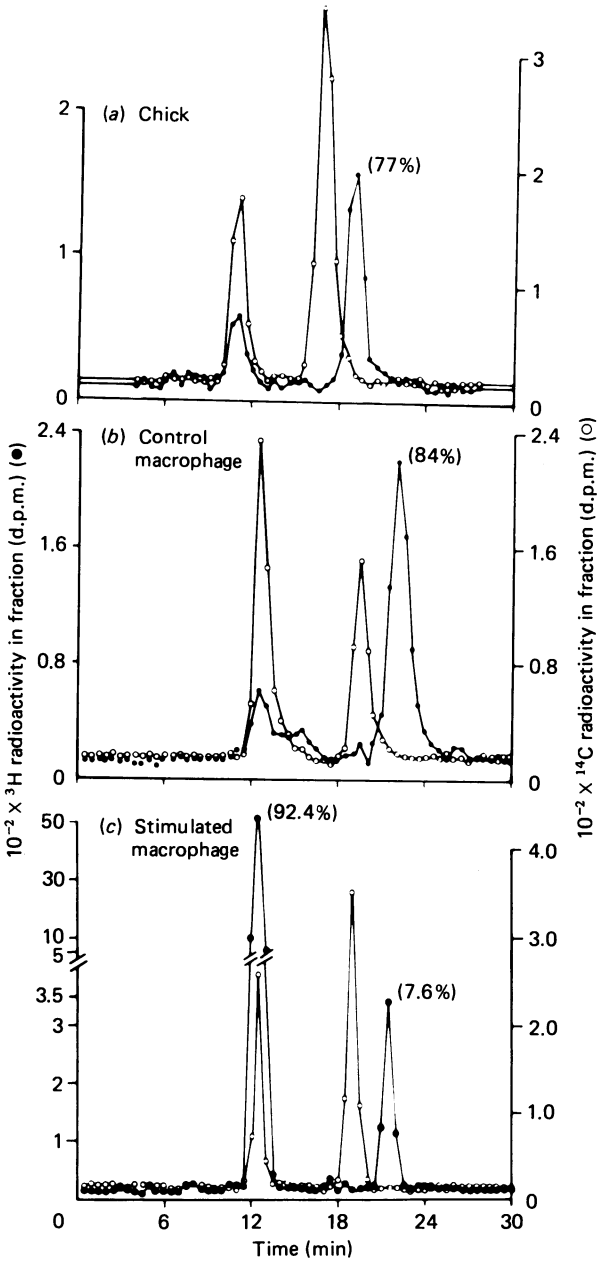

Fig. 6. [^{3}H]Polyols derived from [^{3}H]InsP_4 fractions of chick erythrocytes and mouse macrophages

[^{3}H]InsP_4 fractions were isolated from [^{3}H]Ins-labelled chick erythrocytes and from both unstimulated and PAF-stimulated (2 μg/ml, 40 s) [^{3}H]Ins-labelled macrophages, as described in the Materials and methods section (see also the legend to Fig. 4). Each [^{3}H]InsP_4 fraction was then subjected to periodate oxidation, reduction and dephosphorylation to yield [^{3}H]polyols. During this procedure, each sample was 'spiked' with a mixture of polyol standards, including D-[^{14}C]glucitol and *myo*-[^{14}C]inositol (see the Materials and methods section). The [^{3}H]- and [^{14}C]-polyols present were identified by h.p.l.c., using a Brownlee Polypore-Ca column as described in the Materials and methods section. The flow rate was 0.2 ml/min. Fractions (0.5 min) of the column eluate were collected and their ^{14}C and ^{3}H content measured by liquid scintillation counting. The proportions of starting ^{3}H and ^{14}C radioactivity recovered in the radioactivity polyols are quoted for each individual sample. Similar results were obtained with three independent preparations of each [^{3}H]-InsP_4. (*a*) Chick [^{3}H]InsP_4: recovery of ^{3}H, 65%; recovery of ^{14}C, 69%; (*b*) unstimulated-macrophage [^{3}H]-InsP_4: recovery of ^{3}H, 52%; recovery of ^{14}C, 69%; (*c*) PAF-stimulated-macrophage [^{3}H]InsP_4: recovery of ^{3}H, 58%; recovery of ^{14}C, 68%.

from D-[^{3}H]Ins(1,4,5)P_3 (Irvine *et al.*, 1984; Grado & Ballou, 1961), [^{3}H]altritol from D-[^{3}H]Ins(1,3,4)P_3 (Irvine *et al.*, 1984) and [^{3}H]inositol from D-[^{3}H]Ins(1,3,4,5)P_4 (Batty *et al.*, 1985). We applied this strategy to [^{3}H]InsP_4 fractions isolated from control and PAF-stimulated macrophages (see above) and from chick erythrocytes (see the Materials and methods section and Fig. 5). There are six possible InsP_4 species that are susceptible to periodate oxidation: D- and L-Ins(1,4,5,6)-P_4, D- and L-Ins(1,2,3,4)P_4, and D- and L-Ins(2,3,4,5)P_4. If each of these inositol phosphates were to be subjected to periodate oxidation, then reduced and dephosphorylated (as described above) they would yield D- and L-iditol, L- and D-altritol and D- and L-glucitol respectively (see Fig. 3). An InsP_4 that does not possess four adjacent phosphate groups but has them placed as two separate pairs, or as a group of three plus one [e.g. D-Ins(1,3,4,5)-P_4] would not be oxidized by periodate and would therefore yield inositol. In each case, most of the radioactivity (> 60%) was recovered in [^{3}H]polyols, which were identified by h.p.l.c. as [^{3}H]inositol and [^{3}H]iditol (Fig. 6). As noted above, [^{3}H]inositol is compatible with the presence of [^{3}H]Ins(1,3,4,5)P_4, whereas [^{3}H]iditol could only arise from either D- or L-Ins(1,4,5,6)P_4 (Fig. 7).

The enantiomeric configuration of the [^{3}H]iditol derived from each preparation of InsP_4 was determined by presenting it as a substrate to L-iditol dehydrogenase. The substrate requirement for this enzyme is a polyol containing a primary hydroxy group at C-1 and secondary hydroxy groups at C-2 and C-4, with the carbinol groups at C-2 and C-4 in the L-configuration relative to C-1 (Smith, 1962); thus it will utilize L-iditol as a substrate, but not D-iditol (see Figs. 8 and 9).

L-Iditol dehydrogenase catalyses a freely reversible reaction between β-NAD$^+$ and L-iditol, yielding L-sorbose and NADH (McCorkindale & Edson, 1954). Consequently the oxidation of L-iditol can be conveniently monitored by measuring the increase in the absorbance of the reaction mixture at 340 nm (by measuring the concentration of iditol in the starting and final mixture with a calibrated differential refractometer it was possible to measure a stoichoimetry of 1 mol of NADH produced:0.97 mol of iditol consumed).

In 100 mM-Tris (pH 8.3 at 22 °C) and 20 mM-β-NAD$^+$, the commercially available preparation of the dehydrogenase displays a K_m for L-iditol of approx. 30 mM and a $V_{max.}$ of about 30 nmol/min per mg of solid. When assayed against 50–100 μM-L- and D-iditol, the preparation oxidized D-iditol at 0.09 times the rate that it oxidized L-iditol (mean of three independent experiments; see Fig. 8 and its legend for further details).

The [^{3}H]iditol derived from both chick erythrocyte and macrophage [^{3}H]InsP_4 was converted into [^{3}H]sorbose (Fig. 10 and Table 1) at the same rate as L-iditol. D-[^{14}C]Iditol, derived from D-[^{14}C]Ins(1,4,5)P_3, was not oxidized by L-iditol dehydrogenase in the same assays. The [^{3}H]iditol is therefore identified as the L-enantiomer, and hence the periodate-sensitive [^{3}H]InsP_4 derived from

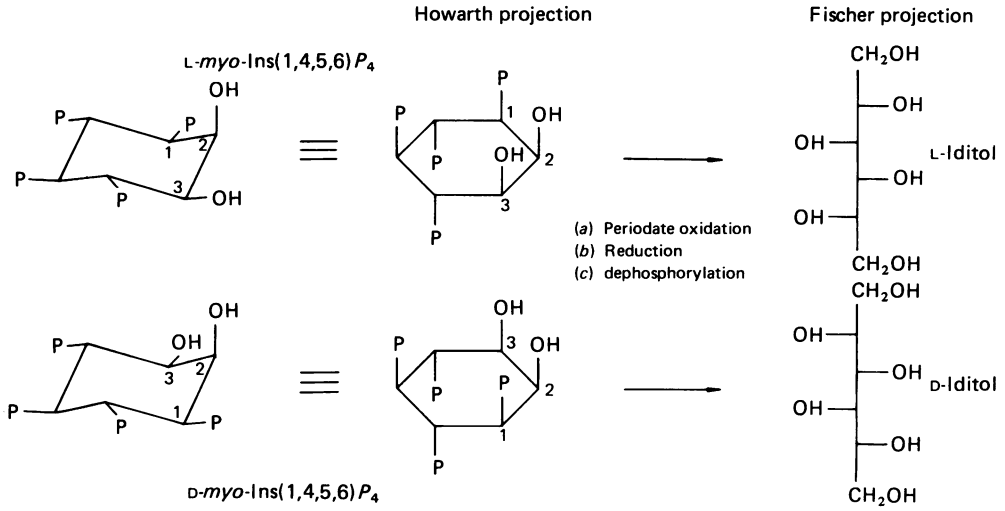

Fig. 7. Periodate oxidation, reduction and dephosphorylation of L- and D-Ins(1,4,5,6)P_4

The abbreviated conformational diagrams of D- and L-Ins(1,4,5,6)P_4 are presented with the numbering system for carbon atoms in the inositol ring indicated. The conventional numbering of the parent *myo*-inositol moiety is such that the carbon at the L-1 substitution site is called 'C-1'. The Haworth projections of these two conformational diagrams are also shown. The polyols derived from the periodate oxidation, reduction and dephosphorylation of these inositol phosphates are drawn in the form of a Fischer projection. It should be emphasized that L- and D-Ins(1,4,5,6)P_4 are stereoisomers, but L-Ins(1,4,5,6)P_4 is, and could be named (though by convention it is not), D-Ins(3,4,5,6)P_4. For clarification, see the reviews by Agranoff (1978) and Parthasarathy & Eisenberg (1986).

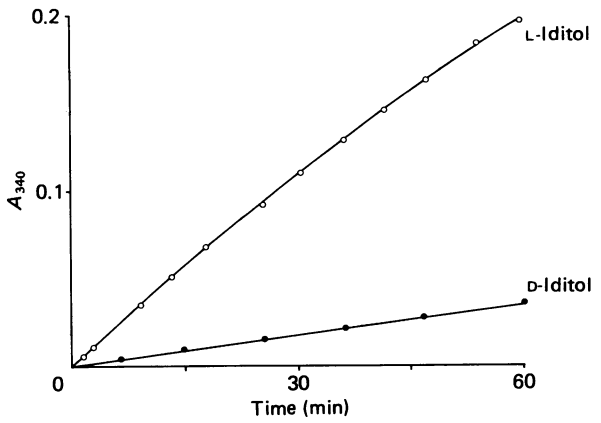

Fig. 8. Oxidation of L- and D-iditol by L-iditol dehydrogenase

L-Iditol dehydrogenase (polyol dehydrogenase; 1 unit/ml) was incubated with either 100 μM-L-iditol (○) or 100 μM-D-iditol (●) (the K_m of the preparation for L-iditol was 30 mM) in 100 mM-Tris/20 mM-β-NAD^+, pH 8.3 (22 °C). The absorbance of the reaction mixture at 340 nm was monitored. At the end of this experiment, 32% of the L-iditol and 3.0% of the D-iditol had been oxidized. The commercially available preparation of L-iditol dehydrogenase oxidized L-iditol 11.1 (mean) times more rapidly than D-iditol (mean of three independent experiments using substrate concentrations in the first-order range).

both avian erythrocytes and mouse macrophages is unambiguously assigned the structure L-Ins(1,4,5,6)P_4.

Most (70–80%) of the [^{3}H]InsP_4 isolated from chick erythrocytes was L-[^{3}H]Ins(1,4,5,6)P_4 (Fig. 6). This result is entirely consistent with a much earlier observation by Johnson & Tate (1969) that the InsP_4 in chicken erythrocytes is susceptible to periodate oxidation and yields iditol upon reduction and dephosphorylation. They did not determine which enantiomer of iditol was made.

Most of the [^{3}H]InsP_4 isolated from unstimulated macrophages was also L-[^{3}H]Ins(1,4,5,6)P_4; in two separate preparations, the proportion of polyol radioactivity recovered in L-iditol was 64 and 84% (Fig. 6; results not shown).

In contrast, [^{3}H]InsP_4 derived from PAF-stimulated macrophages consisted very largely of an isomer(s) which was not susceptible to periodate oxidation (92%;

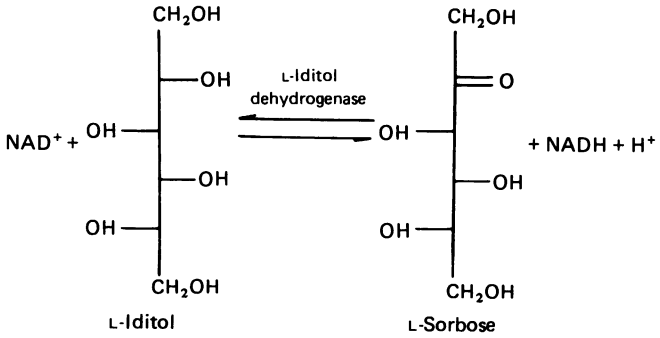

Fig. 9. Oxidation of L-iditol by L-iditol dehydrogenase

L-Iditol dehydrogenase (polyol dehydrogenase; L-iditol:NAD oxidoreductase; 1.1.1.14) catalyses the freely reversible reaction shown above. The essential structural requirements of a polyol for it to act as a substrate for this enzyme are: a primary alcohol at C-1; a secondary alcohol at C-2 that is in an L-configuration relative to C-1; and a secondary alcohol at C-4 that is in an L-configuration relative to C-1.

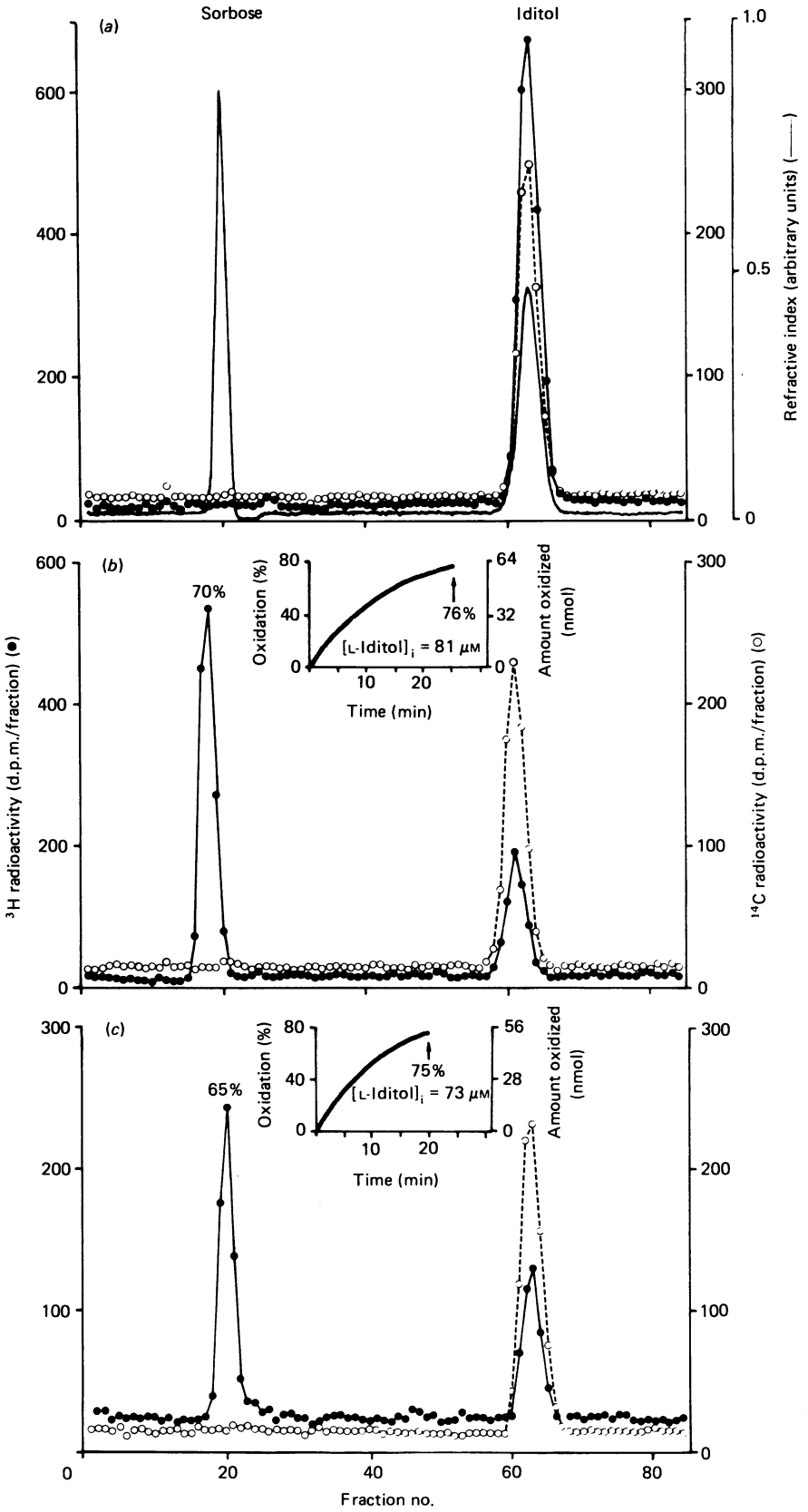

Fig. 10. Oxidation of [^{3}H]iditol, derived from chicken erythrocyte and macrophage [^{3}H]InsP_4, by L-iditol dehydrogenase

D-[^{14}C]Iditol (1000 d.p.m. at a specific radioactivity at about 200 d.p.m.·pmol^{-1}) was mixed with [^{3}H]iditol derived from either avian erythrocyte or macrophage [^{3}H]InsP_4 (1000–3000 d.p.m. at a specific radioactivity which, although unmeasured, would be higher than that of the D-[^{14}C]iditol), L-iditol (final concns. 70–100 μM), 1.5 units of L-iditol dehydrogenase and assay buffer as described in the Materials and methods section. The progress of the reaction was monitored continuously by measuring the absorbance of the reaction mixture of 340 nm and was terminated at various times (1 min, *a*; 25 min, *b*; 19.5 min, *c*) by heat

Table 1. Oxidation of [^{3}H]- and L-iditol by L-iditol dehydrogenase

Incubation conditions were as described in the legend to Fig. 10. $[L\text{-Iditol}]_i$ is the initial concentration of L-iditol.

[^{3}H]Iditol derived from:	$[L\text{-Iditol}]_i$	L-Iditol oxidized (%)	[^{3}H]-Iditol oxidized (%)
Macrophage [^{3}H]InsP_4	40	29	35
Avian erythrocyte [^{3}H]InsP_4	91	35	40

Fig. 6), with only a small proportion (8%; Fig. 6) present as L-Ins(1,4,5,6)P_4. However, since the total amount of radioactivity in the InsP_4 fraction from stimulated cells is approx. 10-fold greater than that in the InsP_4 from control cells, this result is consistent with the notion that PAF stimulates the rapid formation of D-Ins(1,3,4,5)P_4, but does not acutely alter the levels of L-Ins(1,4,5,6)P_4.

DISCUSSION

D-Ins(1,3,4,5)P_4 is synthesized from D-Ins(1,4,5)P_3 by a widely distributed kinase enzyme (Irvine *et al.*, 1986). The K_m [for D-Ins(1,4,5)P_3] and $V_{max.}$ of D-Ins(1,4,5)P_3 kinase are poised such that the rate of production of D-Ins(1,3,4,5)P_4 is highly sensitive to the prevailing cellular levels of its precursor. Hence, when the concentration of D-Ins(1,4,5)P_3 rises during hormone stimulation, that of D-Ins(1,3,4,5)P_4 rapidly follows (Batty *et al.*, 1985; Hawkins *et al.*, 1986). The data in the Results section are entirely consistent with these observations, although they also demonstrate the presence of another inositol tetrakisphosphate in avian and mammalian cells, namely L-Ins(1,4,5,6)P_4. In macrophages, at least, the levels of L-Ins(1,4,5,6)P_4 are insensitive to brief stimulation with PAF.

Johnson & Tate (1969) have reported that the inositol tetrakisphosphate found in chicken erythrocytes yielded iditol upon periodate oxidation, reduction and dephosphorylation. They correctly interpreted this as evidence that the InsP_4 in chicken erythrocytes was D- or L-Ins(1,4,5,6)P_4. Mayr & Dietrich (1987) have recently assigned the proton-, ^{13}C- and ^{31}P-n.m.r. spectra of this compound and confirmed its identity as D- or L-Ins(1,4,5,6)P_4. They suggested that the inositol tetrakisphosphate species found in mammalian and avian cells are different. The data shown above suggest that L-Ins(1,4,5,6)P_4 is the predominant inositol tetrakisphosphate in both avian and unstimulated mammalian cells (although the macrophages were not labelled to isotopic equilibrium, the relative [^{3}H]Ins content of the inositol tetrakisphosphates is very unlikely to be substantially different from their relative concentrations) and, moreover, that both D-Ins(1,3,4,5)P_4 and L-Ins(1,4,5,6)P_4 can be found in the same cells (the macrophage cultures were more than 95% pure; see Whetton *et al.*, 1986).

The function(s) of L-Ins(1,4,5,6)P_4 is currently undefined, although the available data do give some clues concerning its metabolic relationship with the products of agonist-stimulated inositol phospholipid breakdown. The absence of a phosphate in the D-1 substitution site suggests that this compound is not a direct product of the phosphodiesteratic cleavage of any currently recognized inositol phospholipid or the product of a one-step phosphorylation of any of the known 'hormone-sensitive' inositol phosphate species. Moreover, the fact that the levels of L-[^{3}H]Ins(1,4,5,6)P_4 do not appear to change during acute hormone stimulation, whereas those of D-Ins(1,3,4,5)P_4 rise rapidly, implies at most a distant kinship to D-Ins(1,4,5)P_3 or the other products of inositol lipid hydrolysis, or that its synthesis is not strongly controlled by the prevailing concentrations of these compounds. Together these factors suggest that L-Ins(1,4,5,6)P_4 may lie on a metabolic pathway distinct from that involving inositol phospholipid metabolites. Since both chick erythrocytes and macrophages also contain InsP_5 (Fig. 1; Johnson & Tate, 1969), the possibility arises that L-Ins(1,4,5,6)P_4 may be an intermediate in either the synthesis or breakdown of InsP_5 [Chakrabati & Majumber, 1978; Cosgrove, 1980; see Stephens *et al.*, 1988 (the following paper)]. This view is strengthened by the occurrence of substantial quantities of L-Ins(1,4,5,6)P_4 in chick erythrocytes, cells that are in the process of synthesizing large quantities of InsP_5, which appears to replace 2,3-bisphosphoglyceric acid as a modulator of oxyhaemoglobin binding in such cells (Johnson & Tate, 1969). The function of InsP_5 in macrophages is not known at present.

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denaturation. The extent of reaction in terms of the percentage of L-iditol oxidized was determined as described in the Materials and methods section (inset Figures: $[L\text{-iditol}]_i$ is the initial concentration of L-iditol). The proportions of [^{3}H]iditol and D-[^{14}C]iditol oxidized were determined by applying the reaction mixture, after desalting, to a cation-exchange h.p.l.c. column (resin in the Pb^{2+} form) and counting fractions of the eluate (four-drop fractions were collected at roughly 30 s intervals from 5 min after injection) for ^{14}C (○) and ^{3}H (●) radioactivity by application of standard dual-label liquid-scintillation techniques. The positions at which authentic L-iditol and L-sorbose (20 μg of each) were eluted from the column were determined by continuously monitoring the refractive index of the eluate in an independent sample (Fig. 10*a*, continuous line). The results from two independent experiments in which [^{3}H]iditol derived from avian erythrocyte or macrophage [^{3}H]InsP_4 was incubated with L-iditol dehydrogenase (as described above) are shown in Table 1.

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