Biochem. J. (1991) 280, 323–329 (Printed in Great Britain)

Multiple isomers of inositol pentakisphosphate in Epstein–Barr-virus-transformed (T5-1) B-lymphocytes

Identification of inositol 1,3,4,5,6-pentakisphosphate, D-inositol 1,2,4,5,6-pentakisphosphate and L-inositol 1,2,4,5,6-pentakisphosphate

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Substantial amounts of three [^{3}H]InsP_5 isomers were detected in [^{3}H]inositol-labelled human lymphoblastoid (T5-1) cells. Their structures were determined by h.p.l.c. [Phillippy & Bland (1988) Anal. Biochem. **175**, 162–166], and by utilizing a stereospecific D-inositol 1,2,4,5,6-pentakisphosphate 3-kinase from *Dictyostelium discoideum* [Stephens & Irvine (1990) Nature (London) **346**, 580–583]. The structures were: inositol 1,3,4,5,6-pentakisphosphate, D-inositol 1,2,4,5,6-pentakisphosphate and L-inositol 1,2,4,5,6-pentakisphosphate. The relative proportions of these isomers (approx. 73:14:14 respectively) were unaffected by cross-linking anti-IgD receptors. The T5-1 cells also contained InsP_6 and three Ins P_4s, which were identified as the 1,3,4,5, 1,3,4,6 and 3,4,5,6 isomers. In incubations with permeabilized T5-1 cells, both 1,3,4,6 and 3,4,5,6 isomers of InsP_4 were phosphorylated solely to Ins(1,3,4,5,6)P_5. Permeabilized cells also dephosphorylated InsP_6, even in the presence of a large excess of glucose 6-phosphate to saturate non-specific phosphatases. In the latter experiments the following isomers of InsP_5 accumulated: D- and/or L-Ins(1,2,3,4,5)P_5, plus D- and/or L-Ins(1,2,4,5,6)P_5. This demonstration that multiple isomers of InsP_5 may be formed *in vivo* and *in vitro* by a transformed lymphocyte cell line adds a new level of complexity to the study of inositol polyphosphate metabolism and function.

INTRODUCTION

There is now widespread acceptance that the receptor-activated hydrolysis of PtdIns(4,5)P_2 yields Ins(1,4,5)P_3, which mobilizes intracellular Ca^{2+} stores (Berridge & Irvine, 1989). Ins(1,4,5)P_3 can be phosphorylated to Ins(1,3,4,5)P_4, which may contribute to the control of cellular Ca^{2+} fluxes (Berridge & Irvine, 1989). Inositol is salvaged for inositol lipid resynthesis from Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 after their complete dephosphorylation by a group of specialized phosphatases (Shears, 1991). However, we must now add to this well-known cycle of inositol phosphate synthesis and degradation the reactions that lead to the synthesis and metabolism of InsP_5 and InsP_6 (Shears, 1991).

In those mammalian cell types where the structure of InsP_5 has been studied, it was found to be 95–100% Ins(1,3,4,5,6)P_5 [e.g. in bovine brain (Phillippy & Bland, 1988), platelets (Mayr, 1988), liver (Nogimori *et al.*, 1991), HL60 and NG115-401L cells (Stephens *et al.*, 1991), and a pancreatoma cell line (Menniti *et al.*, 1990)]. Ins(1,3,4,5,6)P_5 is also the predominant InsP_5 isomer in chicken erythrocytes (Johnson & Tate, 1969). The only known precursors of Ins(1,3,4,5,6)P_5 are Ins(3,4,5,6)P_4 and Ins(1,3,4,6)P_4 (Stephens *et al.*, 1988*a*,*b*; Balla *et al.*, 1989*a*; Shears, 1989).

Ins(1,3,4,5,6)P_5 and InsP_6 are present in cells at higher concentrations than other inositol phosphates (Shears, 1991), so their synthesis represents a considerable and perhaps even ubiquitous investment of cellular energy stores and enzyme synthesis. It is possible that these polyphosphates can act as extracellular signals (Vallejo *et al.*, 1988; Barraco *et al.*, 1989). However, their intracellular function in mammalian cells is unknown, although a significant role for Ins(1,3,4,5,6)P_5 at least is implicit in observations that its metabolism is regulated by receptor occupation (Tilley *et al.*, 1987; Pittet *et al.*, 1989; Balla *et al.*, 1989*b*; Menniti *et al.*, 1990). Indeed, there is now evidence that Ins(1,3,4,5,6)P_5 is dephosphorylated to Ins(3,4,5,6)P_4 as part of a receptor-regulated futile cycle between these two compounds (Menniti *et al.*, 1990).

We now report that Epstein–Barr-virus-transformed B-lymphocytes contain substantial amounts of multiple isomers of InsP_5. Thus Ins(1,3,4,5,6)P_5 is not the only quantitatively important InsP_5 isomer in all mammalian cell types. This complication will need to be taken into account in future studies of the metabolism of this group of polyphosphates.

MATERIALS AND METHODS

Materials

T5-1 cell culture was a gift from Dr. D. Pious (University of Washington, Seattle, WA, U.S.A.). The monoclonal anti-IgD (δ-TA4.1; see Kubagawa *et al.*, 1982) was a gift from Dr. H. Kubagawa (University of Alabama). D-[^{3}H]Ins(1,2,3,4,6)P_5, D/L-[^{3}H]Ins(1,2,4,5,6)P_5, D/L-[^{3}H]Ins(1,2,3,4,5)P_5, D-[^{3}H]Ins-(1,3,4,5,6)P_5 and D-[^{32}P]Ins(1,2,4,5,6)P_5, and the supernatant

Abbreviations used: inositol phosphates are described according to IUPAC nomenclature with reference to the 1-D phosphate [see Biochem. J. (1989) **258**, 1–2], except for InsP_5 isomers. For these, both D- and L- prefixes are used, so as to highlight enantiomeric relationships (see Stephens *et al.*, 1991). The D/L- prefix is used where there is (or may be) a mixture of enantiomers, but without definition of their relative proportions. In this respect both Ins(1,3,4,5,6)P_5 and Ins(1,2,3,4,6)P_5 are not optically active.

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fractions of *Dictyostelium*, were all prepared as described by Stephens *et al.* (1991). [^{14}C]Ins(1,3,4)P_3 was prepared by h.p.l.c. fractionation of an extract of carbachol-stimulated parotid acinar cells (see Hughes *et al.*, 1989) prelabelled with [^{14}C]inositol (American Radiolabelled Chemicals, St. Louis, MO, U.S.A.). [^{3}H]Ins(3,4,5,6)P_4 and [^{3}H]Ins(1,3,4,5,6)P_5 were isolated from [^{3}H]inositol-labelled and bombesin-stimulated AR42J cells (Menniti *et al.*, 1990). [^{3}H]Ins(1,3,4,6)P_4 was prepared by phosphorylation of [^{3}H]Ins(1,3,4)P_3 with partially purified hepatic 6-kinase (Shears, 1989). [^{32}P]Ins(1,3,4,5)P_4 and [^{32}P]Ins(1,4,5)P_3 (with > 94% of the ^{32}P in the 5-phosphate; results not shown) and [^{3}H]InsP_6 were purchased from New England Nuclear. [^{3}H]Inositol, and ^{3}H-labelled standards of Ins1P, Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 were purchased from Amersham (Arlington Heights, IL, U.S.A.). Type 1 Ins(1,3,4,5)P_4 5-phosphatase was purified as described by Hansen *et al.* (1987) and was kindly given by Dr. K. Nogimori (Calcium Regulation, LCMP, NIEHS, NIH). Sources of other materials are given in Menniti *et al.* (1990).

Incubation and extraction of T5-1 cells

T5-1 cells were labelled for 24 h in RPMI 1640 culture medium supplemented with 5 mg of insulin/ml, 5 mg of transferrin/ml, 5 μg of sodium selenite/ml, 1 g of BSA/l containing 0.5–1.5% linoleic acid (Sigma Chemical Co.), 5 μg of inositol/ml and 5 μCi of [^{3}H]inositol/ml. Before experimentation, cells were collected into centrifuge tubes, spun (5 min at 1000 *g*), washed twice and resuspended at an approximate density of 10^8 cells/ml in 0.5 ml portions of RPMI 1640 culture medium containing 35 μg of inositol/ml, but without [^{3}H]inositol. Where indicated, anti-IgD was added for 10 min (final concn. 10 μg/ml). Samples (300 μl) were quenched with 0.5 ml of ice-cold 20% (w/v) trichloroacetic acid containing sodium phytate (250 μg/ml) plus phytate hydrolysate (25 μg/ml) (Wreggett *et al.*, 1987). Cell extracts were centrifuged for 20 min at 700 *g* and the supernatants were removed. The cell pellet was re-extracted with 0.5 ml of 20% trichloroacetic acid, and the supernatants were combined and neutralized by washing with 5 × 2 ml of water-saturated diethyl ether; 100 μl of 50 mM-EDTA was added during the final wash.

H.p.l.c. analysis of inositol phosphates

Neutralized samples of T5-1 extracts were applied to a 10 cm Partisphere 5 SAX column (Whatman, Clifton, NJ, U.S.A.). The inositol phosphates were then eluted with a gradient obtained by mixing water (Buffer A) with $NH_4H_2PO_4$, pH 3.8, with H_3PO_4 (Buffer B) according to the following protocol: 0–20 min, B increased linearly from 0 to 0.05 M; 20 min, B increased to 0.2 M; 20–30 min, B = 0.2 M; 30 min, B increased to 0.54 M; 30–60 min, B increased linearly to 0.56 M; 60 min, B increased to 0.8 M; 60–75 min, B = 0.8 M; 75 min, B increased to 1.6 M; 75–85 min, B = 1.6 M; 85 min, B increased to 2 M; 85–95 min, B = 2 M. Fractions (0.5 min) were collected and counted for radioactivity. In order to prepare InsP_4 and InsP_5 isomers for desalting and isomeric characterization, ten cell extracts were combined and 0.02% of each h.p.l.c. fraction was counted for radioactivity.

Alternative h.p.l.c. procedures were used in some instances: i.e. an Adsorbosphere 5 μ Sax column eluted with a linear gradient of 0–0.85 M-$(NH_4)_2HPO_4$ (pH 3.35 with H_3PO_4) over 120 min (Balla *et al.*, 1989*b*; Menniti *et al.*, 1990); a Partisphere 5 μ SAX column eluted with $(NH_4)_2HPO_4$, pH 3.8, with H_3PO_4 (Stephens *et al.*, 1991); an Ion-Pac AS7 column (see below).

Desalting of inositol phosphates

InsP_4 isomers were separated from the salt in the h.p.l.c. eluate by using NH_4HCO_3 (Shears *et al.*, 1988) with recoveries of 70–75%. InsP_5 isomers were desalted with HCl (Shears, 1989), and the recovery of both Ins(1,3,4,5,6)P_5 and D/L-ins(1,2,4,5,6)P_5 through these procedures was 70–75%.

Structural identification of inositol phosphates

Alkaline hydrolysis of Ins(1,3,4,6)P_4 was performed as described by Shears (1989). This procedure results in dephosphorylation without phosphate migration (Tomlinson & Ballou, 1961), and the mixture of inositol monophosphates produced was identified by the h.p.l.c. separation described elsewhere (Stephens *et al.*, 1988*a*), except that the isocratic elution was from 5–130 min with 24% 0.2 M-sodium acetate (pH 3.75 with acetic acid). Typical peak elution times, determined with appropriate standards (Stephens *et al.*, 1988*a*; Menniti *et al.*, 1990), were as follows: Ins1P/Ins3P = 58 min; Ins2P = 78 min; Ins5P = 113 min; Ins4P/Ins6P = 122 min.

Under appropriate conditions, inositol polyphosphates are oxidized only at vicinal hydroxyl groups by periodate, and after reduction and dephosphorylation the resultant polyol is indicative of the structure of the original polyphosphate (Grado & Ballou, 1961). Periodate treatment, reduction and dephosphorylation of 5000–10000 d.p.m. of [^{3}H]InsP_4 was performed as described by Stephens *et al.* (1988*a*), with minor modifications (Shears, 1989). The resultant [^{3}H]polyols were identified by h.p.l.c. on a Polybore-Pb column as described elsewhere (Stephens *et al.*, 1988*a*). In some experiments, the h.p.l.c. fractions that contained [^{3}H]iditol were freeze-dried, and the enantiomeric composition of iditol was determined by using a stereospecific polyol dehydrogenase (see the Results section and Stephens *et al.*, 1988*a*).

The incubations used to quantify [^{3}H]Ins(1,3,4,5)P_4 with the purified Ins(1,3,4,5)P_4 5-phosphatase and the methodology for perchlorate-quench and Freon/octylamine neutralization are described by Menniti *et al.* (1990). Samples were analysed by h.p.l.c. on an Adsorbosphere SAX column (see above).

The h.p.l.c. separation of InsP_5 isomers was a modification of the technique described by Phillippy & Bland (1988). The chromatographic system was an Ionpac AS7 h.p.l.c. column, which was preceded by an Ionpac AG7 guard column (Dionex Corp., Sunnyvale, CA, U.S.A.), eluted at a flow rate of 0.5 ml/min. Each day the columns were initially pre-equilibrated for 1 h with 0.15 M-HNO_3, followed by 12 min with water. Samples were injected in a volume of 20 μl of water containing 1 μmol of P from a phytate hydrolysate (this was prepared by heating a solution of 8.3 mM-ammonium phytate in 10 M-NH_3 for 24 h at 110 °C in a sealed glass tube, and then the ammonia was removed by freeze-drying): 1 min fractions were collected. After 1 min the elution medium switched to 0.15 M-HNO_3/15 mM-$(NH_4)_2HPO_4$. The elution medium was returned to water after 50 min, and after a further 12 min the next sample was injected. All solutions were made fresh each day, degassed by sparging with helium, and were stored in plastic beakers which had been pre-washed with 1 M-HNO_3. Recoveries of [^{3}H]InsP_5 from the column were 75–80%. In excess of 50 runs could be made on a single column without loss of resolution.

The enantiomeric composition of D/L-[^{3}H]Ins(1,2,4,5,6)P_5 was determined by incubating it, in duplicate, with a 100000 *g* soluble fraction of *Dictyostelium*, which phosphorylates D-Ins(1,2,4,5,6)P_5 but not L-Ins(1,2,4,5,6)P_5 (Stephens & Irvine, 1990; Stephens *et al.*, 1991). The D/L-[^{3}H]Ins(1,2,4,5,6)P_5 for these experiments was first separated from [^{3}H]Ins(1,3,4,5,6)P_5 on a Partisphere 5 μ SAX column (see above) and then desalted by using HCl (see above). Some 3000–4000 d.p.m. of ^{3}H was incubated with 250 μl of medium containing 25 mM-Hepes (pH 7), 5 mM-MgATP, 1 mM-EGTA, 1 mM-$MgCl_2$, 1 mM-dithiothreitol, 1 mg of BSA/ml and approx. 600 d.p.m. of a 1:1 racemic mixture of D- and L-[^{32}P]Ins(1,2,4,5,6)P_5. After 25 min at 25 °C, reactions were quenched with $HClO_4$ and neutralized (see

below). The products were analysed by h.p.l.c. using a Partisphere 5 μ SAX column (see above). Typically, between 30 and 44% of the racemic mixture of D- and L-[^{32}P]Ins(1,2,4,5,6)P_5 was phosphorylated to [^{32}P]InsP_6. By extrapolating the reaction to the point where 50% of the racemic mixture would have been phosphorylated (i.e. all of the D-enantiomer), the proportion of D-[^{3}H]Ins(1,2,4,5,6)P_5 in the original ^{3}H-labelled sample could be estimated.

Metabolism of [^{3}H]inositol phosphates by permeabilized cells

T5-1 cells, suspended to a density of about 2×10^8 cells/ml in buffer containing 100 mM-KCl, 1 mM-$MgCl_2$ and 10 mM-Hepes (pH 7.2 with NaOH), were permeabilized by the addition of 0.02 vol. of saponin (final concn. 0.2 mg/ml), followed immediately by freezing in liquid nitrogen. When required, samples of cells were thawed and incubated with appropriate inositol phosphates at 37 °C as described in the Results section. At various times incubations were quenched with $HClO_4$, neutralized with Freon/octylamine and analysed by h.p.l.c. using an Adsorbosphere SAX column (see above).

RESULTS

Characterization of InsP_5 isomers in T5-1 cells

B-lymphocytes from both mice (Bijsterbosch & Klaus, 1985; Brunswick *et al.*, 1989) and man (Guy *et al.*, 1985; Lane *et al.*, 1990) have been shown to release Ins(1,4,5)P_3 and and mobilize intracellular Ca^{2+} pools when stimulated through surface immunoglobulin. We have investigated the spectrum of inositol phosphates in T5-1 cells, an Epstein–Barr-virus-transformed human lymphoblastoid line (Sato *et al.*, 1972), which imitate the early signal-transduction events of normal human B-cells (F. M. McConnell, unpublished work). Fig. 1 shows a h.p.l.c. profile of an extract of [^{3}H]inositol-labelled cells, fractionated on a Partisphere SAX column. There were multiple peaks of radioactivity, some of which correspond to the elution positions of Ins$P$, Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4. Relatively large proportions of ^{3}H accumulated in Ins$P$, InsP_5 and InsP_6, similarly to other mammalian cell types when they are labelled for 24 h or more (for references, see Shears, 1991).

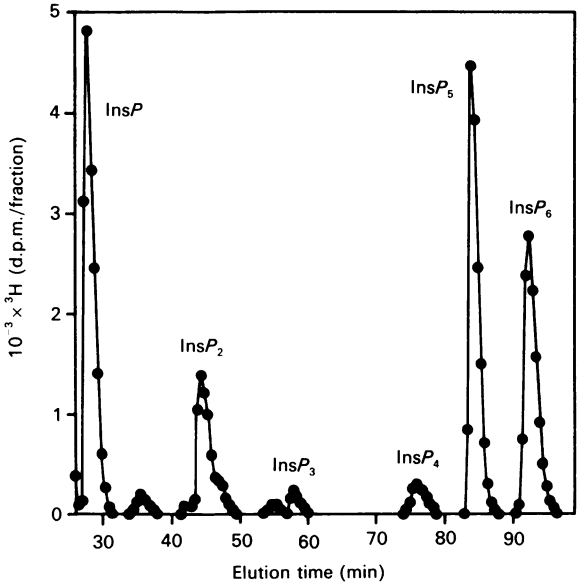

Fig. 1. H.p.l.c. analysis of inositol phosphates in resting T5-1 cells

Cells were labelled for 24 h with [^{3}H]inositol, quenched with trichloroacetic acid and analysed by h.p.l.c. as described in the Materials and methods section. The degree of phosphorylation of each peak was determined from the elution times of ^{3}H-labelled standards run separately before and after the samples: Ins1P, 27 min; Ins(1,4)P_2, 44 min; Ins(1,3,4)P_3, 56 min; Ins(1,4,5)P_3, 59 min; Ins(1,3,4,5)P_4, 79.5 min. For identification of InsP_5 and InsP_6, see the text.

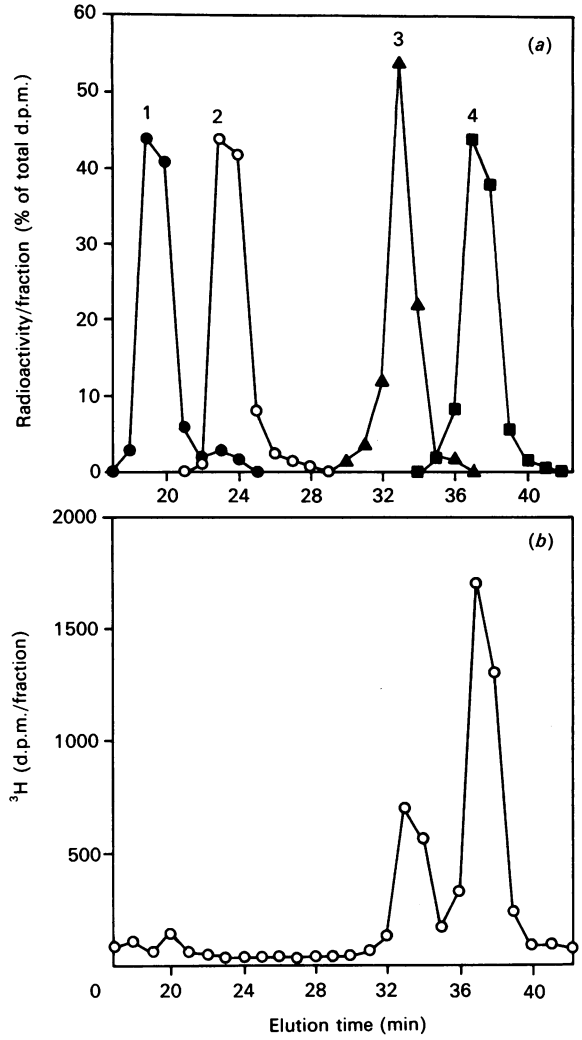

Fig. 2. H.p.l.c. separation of InsP_5 isomers in resting T5-1 cells

(*a*) Chromatography of ^{3}H-labelled standards of InsP_5 (500–1000 d.p.m.; *a*), as described in the Materials and methods section. The standards were each separately applied in individual runs and the data are plotted as percentage of recovered radioactivity (d.p.m.). Peaks: 1, Ins(1,2,3,4,6)P_5; 2, D/L-Ins(1,2,3,4,5)P_5; 3, D/L-Ins(1,2,4,5,6)P_5; 4, Ins(1,3,4,5,6)P_5. Similar profiles were obtained with two additional columns. (*b*) Re-chromatography of approx. 6000 d.p.m. of the T5-1 cell [^{3}H]InsP_5 fraction eluted from a Partisphere SAX column (as in Fig. 1). The Ins(1,3,4,5,6)P_5 peak comprised 73% of recovered radioactivity (d.p.m.). Similar results were obtained with InsP_5 obtained from two additional cell preparations.

The single peak of [^{3}H]InsP_5 from the Partisphere SAX column was rechromatographed on an h.p.l.c. system to further resolve InsP_5 isomers (Fig. 2*a*). The InsP_5 fraction from T5-1 cells was resolved into two components that were identified as D- and/or L-Ins(1,2,4,5,6)P_5 and Ins(1,3,4,5,6)P_5 (Fig. 2*b*). The D/L-Ins(1,2,4,5,6)P_5 peak comprised 27% of total InsP_5 (Fig. 2*b*). Since D/L-Ins(1,2,4,5,6)P_5 is the last of the InsP_5 isomers to be eluted from a Partisphere SAX column (Fig. 5, and see Stephens

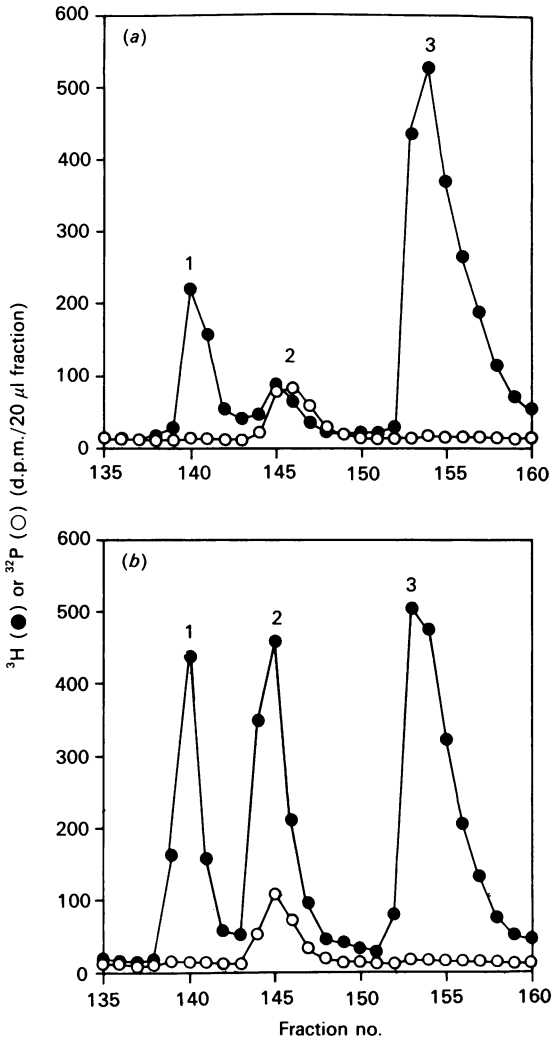

Fig. 3. H.p.l.c. separation of InsP_4 isomers in T5-1 cells

The [^{3}H]InsP_4 fraction from either resting T5-1 cells, or those stimulated for 10 min with anti-IgD, was eluted from a Partisphere SAX column (see Fig. 1) and desalted. Approx. 65000 d.p.m. of [^{3}H]InsP_4 fraction from resting cells (*a*: ●) or about 90000 d.p.m. from stimulated cells (*b*: ●) were each spiked with approx. 6000 d.p.m. [^{32}P]Ins(1,3,4,5)P_4 (○) and re-chromatographed on an Adsorbosphere SAX column as described in the Materials and methods section. Fractions of volume 500 μl were collected, from which 20 μl samples were counted for radioactivity to locate the peaks, which were then separately combined and desalted for further analysis (peak 1, fractions 139–142; peak 2, fractions 144–147; peak 3, fractions 153–160). In resting cells the proportion of ^{3}H in peaks 1:2:3 was 16:9:76. The value obtained from stimulated cells was 21:30:48.

et al., 1991), the ^{3}H-labelled peak which is eluted later (Fig. 1) was identified as InsP_6.

In order to determine the enantiomeric composition of the D/L-[^{3}H]Ins(1,2,4,5,6)P_5 peak in T5-1 cells, it was purified and incubated with a stereospecific D-Ins(1,2,4,5,6)P_5 3-kinase (Stephens *et al.*, 1991; see the Materials and methods section). These incubations also contained an internal standard of a 1:1 racemic mixture of D- and L-[^{32}P]Ins(1,2,4,5,6)P_5. Two preparations of D/L-[^{3}H](1,2,4,5,6)P_5 were analysed. Either 30% or 41% of total ^{3}H-labelled material was phosphorylated to [^{3}H]InsP_6; in each preparation, identical amounts of [^{32}P]InsP_5 were also phosphorylated (results not shown). Thus there must have been a 1:1 racemic mixture of D-[^{3}H]Ins(1,2,4,5,6)P_5 and L-[^{3}H]Ins(1,2,4,5,6)P_5 in both samples, each of which therefore accounted for approx. 14% of total [^{3}H]InsP_5 (see Fig. 2*b*).

We do not believe that the appearance of relatively large amounts of D/L-Ins(1,2,4,5,6)P_5 in T5-1 cells was the result of significant acid-catalysed migration from the 1-phosphate of Ins(1,3,4,5,6)P_5 to the 2-position (see Pittet *et al.*, 1989), even though we used trichloroacetic acid to extract the inositol phosphates from the cells, and HCl was used to desalt the InsP_5 (see the Materials and methods section). Two considerations led to this conclusion. First, the desalting procedures are used routinely in this laboratory, and no measurable conversion of Ins(1,3,4,5,6)P_5 into D/L-Ins(1,2,4,5,6)P_5 has ever been observed (Menniti *et al.*, 1990; results not shown). Second, in control experiments [^{3}H]Ins(1,3,4,5,6)P_5 was taken through the trichloroacetic acid-extraction and neutralization procedures described in the Materials and methods section, and no more than 1% was converted into D/L-[^{3}H]Ins(1,2,4,5,6)P_5 (results not shown). In fact, if Ins(1,3,4,5,6)P_5 was subjected to conditions far more extreme than those used routinely, namely incubation in the acid-quenched medium for 20 h at room temperature before neutralization, only 3% was converted into D/L-Ins(1,2,4,5,6)P_5 (results not shown). Thus we conclude that essentially all the D/L-Ins(1,2,4,5,6)P_5 that we have detected in T5-1 cells is genuinely present in intact cells.

It is widely accepted that the hydrolysis of inositol lipids is initiated after antigen-receptor cross-linking (Klaus *et al.*, 1987). Therefore preliminary experiments were conducted in order to determine if the proportions of InsP_5 isomers in T5-1 cells were affected by cross-linking surface IgD for up to 10 min. Although T5-1 cells responded to this treatment by severalfold increases in levels of Ins(1,3,4,5)P_4 (see below), there was no significant effect on (*a*) absolute levels of [^{3}H]InsP_5, or (*b*) the proportion of ^{3}H distributed between the three InsP_5 isomers, or (*c*) the ratio of InsP_5/InsP_6 (results not shown). These data are indicative of a certain metabolic independence of InsP_5 isomers from the inositol phosphates closely associated with phospholipase C activation.

Characterization of InsP_4 isomers in T5-1 cells

The unusual spectrum of InsP_5 isomers in T5-1 cells led us to investigate the relationship this might have to the pattern of InsP_4 isomers. These studies were also undertaken to obtain information on the origins of the InsP_5 isomers. The InsP_4 fraction from T5-1 cells was usually eluted from the Partisphere SAX column as two peaks, although sometimes only one peak was observed. In any case, the entire InsP_4 fraction was combined, desalted, and rechromatographed on an Absorbosphere SAX column, with an internal [^{32}P]Ins(1,3,4,5)P_4 standard. In extracts from resting T5-1 cells, three [^{3}H]InsP_4 peaks were observed (Fig. 3*a*) which respectively were eluted before, with and after the [^{32}P]Ins(1,3,4,5)P_4 standard. The same three peaks were observed in InsP_4 extracts from T5-1 cells stimulated with anti-IgD for 10 min (Fig. 3*b*), although their relative proportions were altered, owing to a 3.5-fold increase in absolute levels of peak 1, a 6-fold increase in peak 2, and no significant change to levels of peak 3.

The strategies used to analyse the InsP_4 isomers further were those successfully employed in an earlier study (Menniti *et al.*, 1990). First, the [^{3}H]InsP_4 comprising peak 1 in Fig. 3 was expected to contain Ins(1,3,4,6)P_4, on the basis of its earlier elution in relation to [^{32}P]Ins(1,3,4,5)P_4. This putative Ins(1,3,4,6)P_4 was subjected to alkaline hydrolysis, which results in the progressive elimination of phosphate groups (sufficient material for this analysis was only available from stimulated cells). Approx. 1300 d.p.m. of the resultant mixture of [^{3}H]inositol monophosphates was analysed by h.p.l.c. as described in the Materials and methods section. In the two such experiments

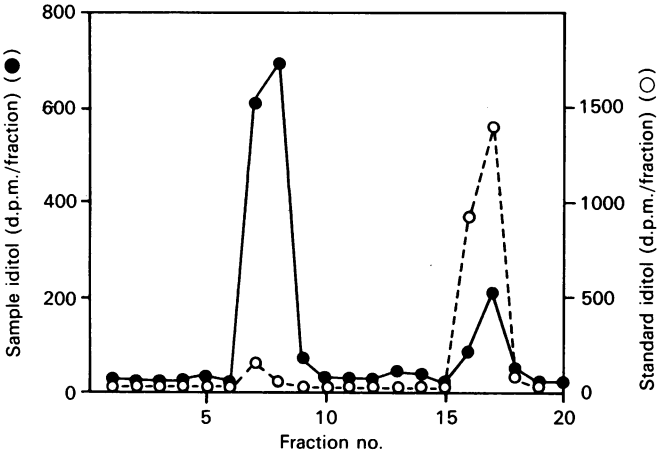

Fig. 4. Determination of the enantiomeric composition of D/L-iditol derived from the Ins(3,4,5,6)P_4/Ins(1,4,5,6)P_4 peak in T5-1 cells

The [^{3}H]InsP_4 comprising peak 3 in Fig. 3(*a*) (i.e. from resting cells) was incubated with periodate, reduced and dephosphorylated to D/L-[^{3}H]iditol (see text) as described in the Materials and methods section. Approx. 1700 d.p.m. of D/L-[^{3}H]iditol (●) or 2500 d.p.m. of genuine D-[^{3}H]iditol (○) were each separately incubated for 45 min at 35 °C in 1 ml of medium containing 50 mM-Tris/HCl (pH 8.3), 20 mM-NAD^+, 100 μM-L-iditol and 2 Sigma Units of polyol dehydrogenase. From the change in A_{340} it was estimated that 85% of L-iditol was oxidized (Stephens *et al.*, 1988*a*). Incubations were quenched by boiling for 3 min, de-ionized with Amberlite resin, freeze-dried and chromatographed on a Polybore-Pb column (Stephen *et al.*, 1988*a*). Some 82% of the [^{3}H]iditol of unknown chirality (●) and 7% of the D-[^{3}H]iditol (○) were oxidized to sorbose (the earlier-eluted peak). Identical results were obtained with material derived from stimulated cells.

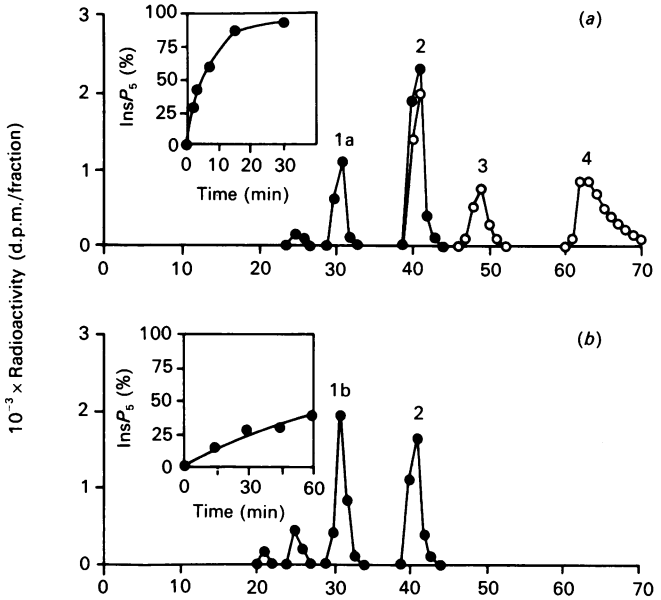

Fig. 5. Phosphorylation of Ins(3,4,5,6)P_4 and Ins(1,3,4,6)P_4 by T5-1 homogenates

Either 8000 d.p.m. of [^{3}H]Ins(3,4,5,6)P_4 (*a*) or 3000–5000 d.p.m. of [^{3}H]Ins(1,3,4,6)P_4 (*b*) were separately incubated with approx. 4×10^6 saponin-permeabilized T5-1 cells in 0.5 ml of medium containing 10 mM-Hepes (pH 7.2 with KOH), 6 mM-$MgSO_4$, 5 mM-ATP, 10 mM-phosphocreatine plus 0.5 mg of phosphocreatine kinase/ml. Reactions were quenched after 0–60 min, neutralized and chromatographed on gravity-fed columns (inset to each panel). Some additional reactions were quenched after 20 min (*a*) or 60 min (*b*) and analysed on a Partisphere SAX column (●) as described in the Materials and methods section. Fig. 5(*a*) also shows the elution positions of standards (○) of [^{3}H]InsP_6 (peak 4), D/L-[^{3}H]Ins(1,2,4,5,6)P_5 (peak 3) and [^{3}H]Ins(1,3,4,5,6)P_5 (peak 2). Peaks 1a and 1b are [^{3}H]Ins(3,4,5,6)P_4 and [^{3}H]Ins(1,3,4,6)P_4 respectively. Similar results were obtained in one additional experiment.

which were performed, only two monophosphate peaks were found, corresponding to Ins1P/Ins3P and Ins4P/Ins6P (results not shown). Ins(1,3,4,6)P_4 is the only InsP_4 that can produce this pattern of monophosphates, and thus this is the only isomer found in peak 1.

That [^{3}H]Ins(1,3,4,5)P_4 comprised peak 2 in Fig. 3 was indicated by its co-elution with an internal [^{32}P]Ins(1,3,4,5)P_4 standard. We further studied this assumption by incubating the putative [^{3}H]Ins(1,3,4,5)P_4 with the stereospecific Ins(1,3,4,5)P_4 5-phosphatase (Cooke *et al.*, 1989). In incubations where > 98% of an internal standard of [^{32}P]Ins(1,3,4,5)P_4 was dephosphorylated, > 95% of the [^{3}H]InsP_4 from either resting or stimulated cells was also hydrolysed to [^{3}H]Ins(1,3,4)P_3, which co-eluted precisely with an internal [^{14}C]Ins(1,3,4)P_3 standard (results not shown). We did not have sufficient material to perform detailed time courses with the 5-phosphatase, although when we adjusted the incubation time so as to metabolize only 31% of [^{32}P]Ins(1,3,4,5)P_4, 29% of [^{3}H]Ins(1,3,4,5)P_4 was also dephosphorylated (one experiments; results not shown). These data indicate that the [^{3}H]InsP_4 in peak 2 is virtually all [^{3}H]Ins(1,3,4,5)P_4. The [^{3}H]InsP_4 that was not attacked by the 5-phosphatase was too small an amount to be further characterized.

The InsP_4 in peak 3 (Fig. 3) was analysed by its incubation with periodate, followed by reduction and dephosphorylation (Menniti *et al.*, 1990). In two experiments from stimulated cells, and one from resting cells, 84–91% of the ensuing [^{3}H]polyols were identified as iditol. The remainder were divided about equally between [^{3}H]polyols which were eluted with either the same retention time as xylitol, or 1 min after inositol (results not shown; see also Menniti *et al.*, 1990). There is no InsP_4 which would be expected to yield xylitol, so this is an unknown side product. The [^{3}H]polyol that was eluted close to inositol corresponded to < 4% of total [^{3}H]InsP_4, which prevented its further analysis. However, this too is an unknown side product (Menniti *et al.*, 1990).

The only InsP_4 isomers that could yield iditol after periodate oxidation are Ins(3,4,5,6)P_4 (which would produce L-iditol) and Ins(1,4,5,6)P_4 (which would produce D-iditol). The enantiomeric composition of the [^{3}H]iditol was ascertained by using a stereospecific polyol dehydrogenase (Fig. 4). Under conditions where we estimated that 85% of an internal standard of L-iditol was oxidized, 82% of the [^{3}H]iditol of unknown chirality was also oxidized (Fig. 4). Thus no more than 4% of the total [^{3}H]iditol could have been the D-enantiomer, which is below the level that we believe is detectable with confidence in this type of analysis. Thus T5-1 cells contain Ins(3,4,5,6)P_4, but no significant amounts of Ins(1,4,5,6)P_4.

In summary, the only InsP_4 isomers that we have found to be present in T5-1 cells in significant amounts are Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4; this is now considered to be the usual complement of InsP_4 isomers (see Shears, 1991), and as such is in complete contrast with the unusual spectrum of InsP_5 isomers (see above).

Phosphorylation of Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4 by permeabilized T5-1 cells

Three InsP_4 isomers are present in T5-1 cells, namely Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4 (see above). Clearly, Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 cannot be precursors for either D- or L-Ins(1,2,4,5,6)P_5 in a single-step phosphorylation

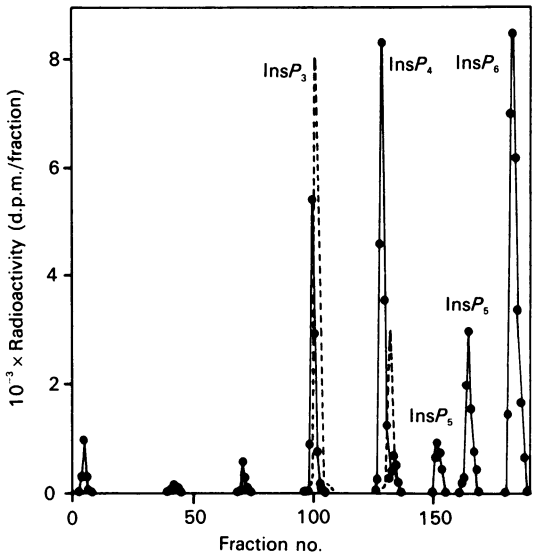

Fig. 6. Analysis of the products of InsP_6 dephosphorylation by permeabilized T5-1 cells

Approx. 150000 d.p.m. of [^{3}H]InsP_6 (12 Ci/mmol, final concn. 120 nM) was incubated for 3 h with approx. 2×10^8 saponin-permeabilized T5-1 cells in 0.6 ml of medium containing 100 mM-KCl, 1 mM-$MgCl_2$, 10 mM-Hepes (pH 7.2 with NaOH) and 18 mM-glucose 6-phosphate. Reactions were quenched, neutralized and chromatographed on an Adsorbosphere SAX h.p.l.c. column as described in the Materials and methods section, with internal standards of [^{32}P]Ins(1,4,5)P_3 and [^{32}P]Ins(1,3,4,5)P_4. Fractions of volume 500 μl were collected, and were counted for ^{3}H (●) and ^{32}P (---; data points omitted for clarity). The ratio of the two InsP_5 isomers was 1:3. In three additional experiments, the earlier-eluted minor InsP_5 comprised 27–29% of total InsP_5. The structures of the two InsP_5 isomers were determined as in Fig. 2; the minor peak was D/L-Ins(1,2,3,4,5)P_5, and the major peak was D/L-Ins(1,2,4,5,6)P_5.

reaction. We therefore investigated the possibility that Ins(3,4,5,6)P_4 [i.e. L-Ins(1,4,5,6)P_4] might at least be a precursor for L-Ins(1,2,4,5,6)P_5. In addition, we compared the ability of T5-1 cells to phosphorylate both Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4 (Fig. 5), since both have been reported to be precursors of Ins(1,3,4,5,6)P_5 in some other cells (Stephens *et al.*, 1988*a*,*b*; Balla *et al.*, 1989*a*; Shears, 1989). We confirmed that both Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4 were phosphorylated to Ins(1,3,4,5,6)P_5, and under our first-order assay conditions the phosphorylation of Ins(3,4,5,6)P_4 proceeded at the faster rate (insets to Fig. 5). In these experiments neither D/L-Ins(1,2,4,5,6)P_5 nor InsP_6 was formed (Fig. 5). In additional 60 min incubations containing the same number of cells plus 10000 d.p.m. of [^{3}H]Ins(1,3,4,5,6)P_5, there was also no detectable formation of D/L-Ins(1,2,4,5,6)P_5 or InsP_6 (results not shown). Thus the origin of InsP_6 remains enigmatic. Note that these experiments (Fig. 5) were analysed on a Partisphere SAX column, rather than the Ion-Pac column, since in the latter case InsP_6 was eluted with considerable peak spreading and poor recovery (results not shown).

Dephosphorylation of InsP_6 by permeabilized T5-1 cells

Since we were unable to identify a potential InsP_4 precursor of either D- or L-Ins(1,2,4,5,6)P_5, we next investigated if InsP_6 was a candidate, since there are substantial amounts of this material in T-1 cells (Fig. 1). Permeabilized T5-1 cells were therefore incubated with 120 nM-[^{3}H]InsP_6, in the presence of 18 mM glucose 6-phosphate to limit the activity of non-specific phosphatases. However, the glucose 6-phosphate did not affect either the metabolism of InsP_6 or the rate of accumulation of InsP_5 (results not shown). Fig. 6 shows a typical result from a 3 h incubation: about 60% of the InsP_6 was metabolized. The first-order rate constant for this reaction was about $1 \times 10\ s^{-1} \cdot (\text{mg of protein})^{-1}$. Two Ins$P_5$ peaks were observed, in the ratio of about 1:3, and together these accounted for about 15% of total radioactivity (Fig. 6). The smaller InsP_5 peak was identified as D/L-Ins(1,2,3,4,5)P_5, and the predominant InsP_5 was found to be D/L-Ins(1,2,4,5,6)P_5 (see the legend to Fig. 6). Other products included InsP_4, InsP_3, InsP_2, and Ins$P$ and Ins, but not Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4, as indicated by the inclusion of internal ^{32}P-labelled standards (Fig. 6).

DISCUSSION

The most important finding in this study has been the identification of considerable proportions of three InsP_5 isomers in a cell of mammalian origin. One of these InsP_5 isomers was identified as Ins(1,3,4,5,6)P_5, which is a well-known intracellular metabolite. The other isomers were identified as D-Ins(1,2,4,5,6)P_5 and L-Ins(1,2,4,5,6)P_5. The occurrence of both enantiomers was established by using a stereospecific 3-kinase prepared from *Dictyostelium* (Stephens & Irvine, 1990; Stephens *et al.*, 1991). A practical consequence of these observations relate to determinations that in some cells Ins(1,3,4,5,6)P_5 metabolism may be receptor-activated (Pittet *et al.*, 1989; Balla *et al.*, 1989*b*; Menniti *et al.*, 1990). The search for a wider applicability of these observations, and its physiological significance, will now require characterization of individual InsP_5 isomers in order to assess specifically the Ins(1,3,4,5,6)P_5 responses.

The relative proportions of these multiple [^{3}H]InsP_5 isomers were assessed in cells labelled for 24 h with [^{3}H]inositol, during which time substantial amounts of label accumulate into InsP_5 and InsP_6 (Fig. 1). [^{3}H]Ins(1,3,4,5,6)P_5 accounted for 73% of total [^{3}H]InsP_5. D-[^{3}H]Ins(1,2,4,5,6)P_5 and L-[^{3}H]Ins(1,2,4,5,6)P_5 respectively each accounted for approx. 14% of total [^{3}H]InsP_5. It was particularly striking that these two ^{3}H-labelled enantiomers were present in exactly equal proportions, although we cannot make absolute comparisons of their masses, since we are not sure that each was labelled to equilibrium. Nevertheless, the identification of substantial amounts of [^{3}H]inositol-labelled D- and L-Ins(1,2,4,5,6)P_5 in a mammalian cell type is novel; previous characterizations of InsP_5 isomers in [^{3}H]inositol-labelled cells (Balla *et al.*, 1989*a*,*b*; Menniti *et al.*, 1990; Stephens *et al.*, 1991; Nogimori *et al.*, 1991) have indicated that at least 95% of total InsP_5 was Ins(1,3,4,5,6)P_5. Even mass analyses have failed to find significant amounts of isomers other than Ins(1,3,4,5,6)P_5 (Mayr, 1988; Phillippy & Bland, 1988).

None of our data indicate that D- and L-Ins(1,2,4,5,6)P_5 were derived by direct phosphorylation of an InsP_4 (see the Results section). In fact, the InsP_4 isomers that we have found to be present in T5-1 cells comprise what is now believed to be a normal complement (Shears, 1991). This is in striking contrast with the unusual spectrum of InsP_5 isomers, the metabolism of which is presumably controlled to an extent that prevents the accumulation of significant levels of atypical InsP_4 isomers.

The metabolic origin of D/L-Ins(1,2,4,5,6)P_5 may be InsP_6, since we found that InsP_6 could be dephosphorylated to D/L-Ins(1,2,4,5,6)P_5 by permeabilized T5-1 cells (Fig. 6). The dephosphorylation of InsP_6 *in vitro* was not specific, since D/L-Ins(1,2,3,4,5)P_5 was also formed (Fig. 6), even though a vast excess of glucose 6-phosphate was present in our assays in an effort to saturate non-specific phosphatases. Since D/L-Ins(1,2,3,4,5)P_5 was not detected in intact cells, our experiments *in vitro* may not precisely mimic the situation *in vivo*. However, in intact cells the prevailing levels of several InsP_5 isomers may be

determined by the relative rates of InsP_6 dephosphorylation and InsP_5 phosphorylation (Stephens *et al.*, 1991). In any case, InsP_6 remains the only cell constituent which we have been able to show may be the precursor for D/L-Ins(1,2,4,5,6)P_5. The absence of a precise specificity of InsP_6 dephosphorylation by permeabilized T5-1 cells resembles the activity of a highly purified hepatic InsP_6 phosphatase (Nogimori *et al.*, 1991). Our experiments with T5-1 cells indicate that there are circumstances where relatively non-specific InsP_6 metabolism may be physiologically significant.

If 'futile cycles' between InsP_6 and several InsP_5 isomers (Stephens *et al.*, 1991) are quite widespread, then in most cell types investigated to date the putative cycles must be poised greatly in favour of InsP_6 synthesis, thus avoiding the accumulation of multiple InsP_5 isomers. Our experiments may indicate that in T5-1 cells the regulation of these putative cycles is significantly different. It is unclear whether this reflects the lymphoid origin of the cell, or it being transformed. Nevertheless, our observations presage a new complexity in the field of inositol polyphosphate metabolism, which may have important ramifications in cell physiology.

We are very grateful to Andy Letcher for performing the InsP_5 3-kinase assays. We also thank Dr. F. Menniti and Dr. K. Oliver for their helpful comments on the manuscript. Part of this work was funded by a U.S. Public Health Service Grant RR00166. L.R.S. is a Babraham Research Fellow.

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28 May 1991/12 July 1991; accepted 17 July 1991