

## Do mammals make all their own inositol hexakisphosphate?

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A highly specific and sensitive mass assay for inositol hexakisphosphate (InsP<sub>6</sub>) was characterized. This centres around phosphorylating InsP<sub>6</sub> with [<sup>32</sup>P]ATP using a recombinant InsP<sub>6</sub> kinase from *Giardia lamblia*, followed by HPLC of the <sup>32</sup>P-labelled products with an internal [<sup>3</sup>H]InsP<sub>7</sub> standard. This assay was used to quantify InsP<sub>6</sub> levels in a variety of biological samples. Concentrations of InsP<sub>6</sub> in rat tissues varied from 10–20 μM (assuming 64 % of wet weight of tissue is cytosol water), whereas using the same assumption axenic *Dictyostelium discoideum* cells contained 352 ± 11 μM InsP<sub>6</sub>. HeLa cells were seeded at low density and grown to confluence, at which point they contained InsP<sub>6</sub> levels per mg of protein similar to rat tissues. This amounted to 1.952 ± 0.117 nmol InsP<sub>6</sub> per culture dish, despite the cells being grown in serum shown to contain no detectable (less than 20 pmol per dish) InsP<sub>6</sub>. These results demonstrate

that mammalian cells synthesize all their own InsP<sub>6</sub>. Human blood was analysed, and although the white cell fraction contained InsP<sub>6</sub> at a concentration comparable with other tissues, in serum and platelet-free plasma no InsP<sub>6</sub> was detected (< 1 nM InsP<sub>6</sub>). Human urine was also examined, and also contained no detectable (< 5 nM) InsP<sub>6</sub>. These results suggest that dietary studies purporting to measure InsP<sub>6</sub> at micromolar concentrations in human plasma or urine may not have been quantifying this inositol phosphate. Therefore claims that administering InsP<sub>6</sub> in the diet or applying it topically can produce health benefits by increasing extracellular InsP<sub>6</sub> levels may require reassessment.

**Key words:** dietary phytate, inositol hexakisphosphate (InsP<sub>6</sub>), mass assay, phytate, phytic acid.

### INTRODUCTION

InsP<sub>6</sub> was originally discovered as the major plant phosphate storage compound [1,2], hence its alternative name, phytic acid. It was generally assumed to be plant specific until the rekindled interest in inositol phosphates that followed the discovery of Ins(1,4,5)P<sub>3</sub> as a second messenger [3] led to the realization that InsP<sub>6</sub> is also found in animal cells (e.g. [4,5] and see [6,7] for reviews).

In the intervening years, the route by which animals synthesize InsP<sub>6</sub> was controversial [6], but further studies have led to a likely consensus. Unlike slime moulds [8] and plants [9], which can synthesize InsP<sub>6</sub> by stepwise phosphorylation directly from inositol, animals apparently incorporate the first three phosphates while the inositol is in its lipid form. That is, Ins(1,4,5)P<sub>3</sub> is formed by phospholipase C action on PtdIns(4,5)P<sub>2</sub>, and Ins(1,4,5)P<sub>3</sub> is then phosphorylated up to InsP<sub>6</sub>. The synthesis of Ins(1,3,4,5,6)P<sub>5</sub> from Ins(1,4,5)P<sub>3</sub> is most likely to be mediated by a two-step conversion catalysed by a single enzyme, Ipk2 (inositol polyphosphate multikinase) [10–12]; this enzyme is essential for mammalian embryonic survival [13], though it is not certain yet whether that is because of its activity in converting Ins(1,4,5)P<sub>3</sub> into Ins(1,3,4,5,6)P<sub>5</sub>. The alternative route from Ins(1,4,5)P<sub>3</sub> to Ins(1,3,4,5,6)P<sub>5</sub> via Ins(1,3,4,5)P<sub>4</sub>, Ins(1,3,4)P<sub>3</sub> and Ins(1,3,4,6)P<sub>4</sub> is probably quantitatively minor, at least in fibroblasts, as suggested by the results of Leyman et al. [14], who showed that mouse embryonic fibroblasts devoid of any Ins(1,4,5)P<sub>3</sub> 3-kinase isoforms synthesized InsP<sub>6</sub> at a rate indistinguishable from wild-type fibroblasts; in contrast, fibroblasts derived from Ipk2 knockout mice show a greatly compromised InsP<sub>6</sub> synthesis [13]. The final step of InsP<sub>6</sub> synthesis (as in plants and slime moulds [8,9]) is the 2-phosphorylation of Ins(1,3,4,5,6)P<sub>5</sub> by

InsP<sub>5</sub> 2-kinase [11,15], an enzyme which is also essential to viable mammalian development [16].

However, although we now know that mammalian cells can synthesize InsP<sub>6</sub>, how they probably do so, and that it is essential that they do synthesize InsP<sub>6</sub>, the contribution of exogenous InsP<sub>6</sub> to mammalian InsP<sub>6</sub> homeostasis is not so clear. A number of studies have reported increases in InsP<sub>6</sub> levels in tissue and body fluids resulting from the supply of exogenous InsP<sub>6</sub> [17–20], and other work has suggested that InsP<sub>6</sub> added to cell cultures or tissues can impinge on their physiology or pathology [21–23]. As a result of these and other studies there are many claims of therapeutic or other beneficial effects of InsP<sub>6</sub> (e.g. [23,24] and many of these are predicated on the idea that exogenous InsP<sub>6</sub> can directly and significantly influence both extra- and intracellular InsP<sub>6</sub> levels in the body.

In the present study, we have designed and characterized an unambiguous mass assay for InsP<sub>6</sub>. This assay is more time-consuming and complex than others (e.g. [18,25]), but its particular strength is a very high sensitivity and applicability to most sample types. Our results provide some new insights into InsP<sub>6</sub> levels in a number of tissues and address whether mammalian cells can make all their own InsP<sub>6</sub>. Our results also cast doubts on the idea that InsP<sub>6</sub> is directly taken up into human tissues and cells.

### EXPERIMENTAL

#### Reagents

All chemicals and biochemicals used were of AnalaR grade unless specified otherwise. [<sup>3</sup>H]InsP<sub>6</sub> was from PerkinElmer. A [<sup>3</sup>H]InsP<sub>7</sub> standard (probably 5-[PP]InsP<sub>5</sub>, see the Results

Abbreviations used: d.p.m., disintegrations per min; Ipk2, inositol polyphosphate multikinase; TCA, trichloroacetic acid.

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section) was prepared by incubating [ $^3\text{H}$ ]InsP<sub>6</sub> with *Giardia lamblia* InsP<sub>6</sub> kinase under the normal assay conditions (see below for details) such that approx. 50% of the InsP<sub>6</sub> was phosphorylated. The correct nomenclature for the product of InsP<sub>6</sub> phosphorylation by InsP<sub>6</sub> kinase is [PP]InsP<sub>5</sub>, and we have used this nomenclature when necessary for unambiguity (e.g. when specifying isomers). However, for most of the present paper we have used InsP<sub>7</sub> as a simpler alternative. Sources of InsP<sub>5</sub> isomers and *scyllo*-inositol hexakisphosphate were as described previously [26].

### InsP<sub>6</sub> kinase

This is a recombinant enzyme from *G. lamblia*. The sequence of *Giardia* InsP<sub>6</sub> kinase (Genbank<sup>®</sup> Nucleotide Sequence Database accession number AY227443) was amplified from *Giardia* genomic DNA (the gene contains no introns), and then cloned into the hexahistidine bacterial vector pET43A, which fuses the solubility-enhancing bacterial protein NusA to the N-terminus of the fusion protein. The enzyme was purified by talon-bead absorption and imidazole elution as described previously [27]. As discussed previously [27], there is no obvious reason why a recombinant mammalian InsP<sub>6</sub> kinase should not be substituted for the *Giardia* enzyme in this assay, although the high affinity and specificity of the *Giardia* InsP<sub>6</sub> kinase (see the Results section) does make it particularly suitable in this context.

### Extraction protocol

For solid tissue samples, 20–50 mg of tissue was weighed before homogenizing with a ground glass pestle in 180  $\mu\text{l}$  of water. This was rinsed with 70  $\mu\text{l}$  of water and then transferred to a 1.5 ml Eppendorf tube, and 50% (w/v) TCA (trichloroacetic acid) added to give a final concentration of 8.3% (v/v). After vortexing and standing on ice for 30 min, samples were centrifuged at 13 000 g for 15 min in a Microfuge, the supernatant was removed and the pellet was kept for protein determination (see below). The supernatant containing inositol phosphates was washed ten times with water-saturated diethyl ether to remove TCA [28] and finally neutralized with 15  $\mu\text{l}$  of 1:10 (v/v) saturated ammonia. Tubes were spun in a centrifugal vacuum dryer for 5 min at 35°C and then for 30 min at 45°C to remove the ether, before being adjusted to 300  $\mu\text{l}$  with water and the pH checked to ensure it was approx. pH 6.0. For most assays, we took as an aliquot the equivalent of 1 mg of wet weight of starting tissue for the InsP<sub>6</sub> assay.

### InsP<sub>6</sub> mass assay

Samples of tissue extract were incubated in a final volume of 300  $\mu\text{l}$  containing the following: 30  $\mu\text{l}$  of 10 $\times$  assay buffer [500 mM Hepes/NaOH (pH 7.5), 1 M KCl, 50 mM MgCl<sub>2</sub>, 10 mM EGTA and 5 mg/ml BSA], 6  $\mu\text{l}$  of 600  $\mu\text{M}$  2-mercaptoethanol and 10  $\mu\text{l}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP (10  $\mu\text{Ci}$ ; PerkinElmer). After 60 min incubation at 37°C, 45  $\mu\text{l}$  of 0.1 M glucose and 6  $\mu\text{l}$  of hexokinase (20 units, Sigma) were added, and the incubation was continued at 37°C for another 90 min to convert all the remaining radioactivity into [ $^{32}\text{P}$ ]glucose 6-phosphate.

The reaction was stopped by the addition of 0.5 ml of 0.4 M ammonium formate/0.1 M formic acid, and the samples were loaded on to 0.5 ml of Dowex Formate in a BioRad Econo Column (with a disc of Whatman Filter paper number 1 on top of the Dowex resin) [29]. Samples were rinsed on to the column with 4 ml of 0.4 M ammonium formate/0.1 M formic acid, and the column was washed with 10 ml of 0.8 M ammonium formate/

0.1 M formic acid to remove any remaining [ $^{32}\text{P}$ ]glucose 6-phosphate. The higher inositol phosphates were eluted with 2  $\times$  1 ml of 2.5 M ammonium formate/0.1 M formic acid. They were spiked with 600–2000 d.p.m. (disintegrations per min) of [ $^3\text{H}$ ]InsP<sub>7</sub> and loaded on to a Whatman Patisil 10 SAX WCS analytical HPLC column (4.6  $\times$  250 mm, catalogue number 4226-001) or a similar-sized column packed with the same material by Phenomenex.

### HPLC

The column was eluted at 1 ml/min flow rate with the following programme: solution A, water and solution B, 1.2 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (pH 3.8) adjusted with phosphoric acid. The profile gives the percentage of solution B (v/v) added: 0 min, 0%; 5 min, 0%; 30 min, 78%; 75 min, 78%; 76 min, 100%; 81 min, 100%; 82 min, 0%; 102 min, 0%.

Fractions (1 ml) were usually collected between 40 and 75 min (InsP<sub>7</sub> elutes at approx. 60 min) and were counted in a scintillation counter after adding 10 ml of Ultima-Flo AP ammonium phosphate-tolerant scintillation fluid (PerkinElmer). The [ $^3\text{H}$ ] and [ $^{32}\text{P}$ ] windows were calibrated using standards to ensure each isotope was counted independently (spill over between channels was < 1%).

### Protein assay

Pellets from the TCA tissue extracts were dissolved in 4 ml of 8 M urea by sonication (in a Decon ultrasonic bath) and vortexing. The majority of material dissolved, but in samples with a high fat content some fat could remain in suspension and may interfere with the assay (this did not happen in any sample analysed in the present study). Samples were adjusted to a final concentration of 6 M urea, and assayed for protein using the Bio-Rad protein assay, based on a method published previously by Bradford [30], using BSA in 6 M urea as a protein standard.

### Blood fractionation

Blood was taken from unfasted human volunteers who denied having taken aspirin in the preceding 48 h, mixed with acid citrate dextrose (97 mM sodium citrate, 78 mM citric acid and 110 mM D-glucose) and centrifuged at 200 g for 20 min (all centrifugations were performed at 4°C). The supernatant from this centrifugation (platelet-rich plasma) was further centrifuged at 2500 g for 20 min, and the resulting supernatant (platelet-free plasma) was removed and frozen. The pellet from the first centrifugation was resuspended in 25 ml of resuspension buffer [10 mM Hepes/NaOH (pH 7.4), 10 mM glucose, 1 mM MgSO<sub>4</sub>, 145 mM NaCl and 5 mM KCl], and then centrifuged (2500 g for 20 min). The 'buffy coat' (mostly white cells) on top of the pellet was removed, along with the supernatant. The pellet (mostly erythrocytes) was resuspended in 25 ml of resuspension buffer and re-centrifuged (2500 g for 20 min). The small amount of buffy coat on this pellet was removed, along with the supernatant, and the remaining pellet (erythrocytes) was then resuspended in 10 ml of resuspension buffer and frozen. The combined supernatants from the two preceding centrifugations were re-centrifuged and resuspended several times as above (2500 g for 20 min) until no erythrocytes were evident at the bottom of the pellet, at which point the final pellet of mostly white cells was resuspended and frozen.

For preparation of serum, fresh blood was left to stand for 4 h, then centrifuged at 3000 g for 20 min, and the supernatant (serum) was decanted and frozen.

## Human experimentation

All human fluid samples were taken with informed consent of the donors in accordance with the Declaration of Helsinki (2000) of the World Medical Association and with the relevant ethical guidelines of the University of Cambridge. The urine sample was taken from an adult male at 10:30 h, 3 h after a meal consisting of a bowl of breakfast cereal and a cup of coffee, with the subject subsequently having free access to water before the sample was taken.

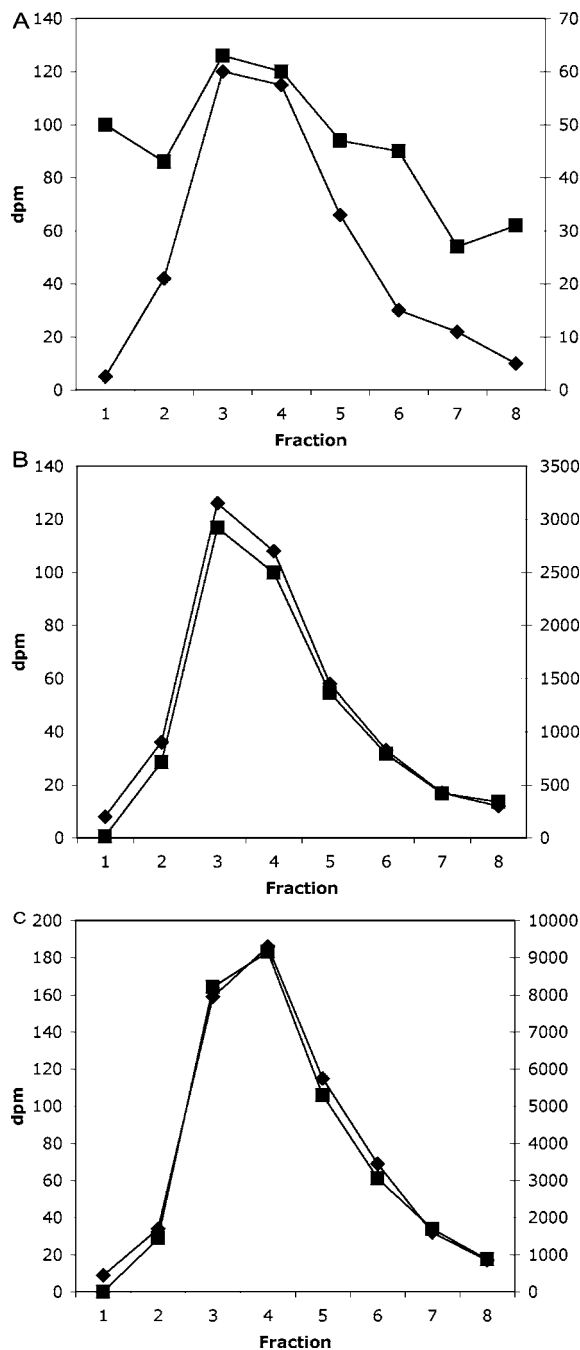
## RESULTS

### Characterization of the enzyme and the assay

Using [ $^3\text{H}$ ]InsP<sub>6</sub>, we established that the *Giardia* kinase has a  $K_m$  of approx. 60 nM (results not shown). We used this enzyme to make our own [ $^3\text{H}$ ]InsP<sub>7</sub> standard for HPLC. Because the *Giardia* gene is closely related to InsP<sub>6</sub> kinases from other eukaryotes, we assumed that its product is 5-[PP]InsP<sub>5</sub> (see [31] for discussion), though we have not established its structure. Draskovic et al. [32] have shown that the mammalian InsP<sub>6</sub> kinases are remarkably specific for the 5-position such that even on prolonged incubation they prefer to add a third phosphate there rather than use another position on the inositol ring. We exposed *bona fide* 5-[PP]InsP<sub>5</sub> synthesized from InsP<sub>6</sub> by a mammalian InsP<sub>6</sub> kinase [a gift from Dr S.B. Shears, Inositol Signaling Group, NIEHS (National Institute of Environmental Health Sciences), Research Triangle Park, NC, U.S.A.] to the *Giardia* enzyme for several hours, but detected no formation of any InsP<sub>8</sub>, which suggests that *Giardia* InsP<sub>6</sub> kinase is a 5-kinase too, and that extra phosphorylation of 5-[PP]InsP<sub>5</sub> is not a complicating factor in the InsP<sub>6</sub> assay.

In the context of this assay, the crucial point is that we always included a [ $^3\text{H}$ ]InsP<sub>7</sub> standard made by the *Giardia* enzyme in every HPLC run, and collected and counted 1 min samples individually for both isotopes. This leads to a precise co-chromatography of the  $^3\text{H}$ -labelled standard and the  $^{32}\text{P}$ -labelled product formed from InsP<sub>6</sub> in the cell extract (e.g. Figure 1); any sample in which this co-chromatography was not absolutely precise was discarded (except for some blanks, where low levels of radioactivity meant that the correlation was less evident: see below). Note that in the Figures, the left-hand axis (◆) is always the  $^3\text{H}$ -labelled InsP<sub>7</sub> standard, and the right-hand axis (■), whose scale varies greatly between Figures (which serves to illustrate the precise co-chromatography of the two isotopes), is the  $^{32}\text{P}$  counts for the same fractions.

We have discussed elsewhere previously [27], and discounted, the possibility of InsP<sub>5</sub> in extracts interfering significantly with the assay. Briefly, InsP<sub>5</sub>s are poor substrates for the *Giardia* enzyme (see below), and any PPInsP<sub>4</sub> products formed elute well before InsP<sub>7</sub> under our HPLC protocol. Any tri-phosphorylated InsP<sub>5</sub> [32] would be formed in minute amounts during our 60 min incubations and also would not precisely co-elute with 5-[PP]InsP<sub>5</sub>. High background levels in the region of the HPLC profile where PPInsP<sub>4</sub> elutes prevented us from extensive quantitative analysis of InsP<sub>5</sub>s as substrates for the *Giardia* InsP<sub>6</sub> kinase. However, in one set of experiments with a fresh batch of [ $\gamma$ - $^{32}\text{P}$ ]ATP (and consequently lower background) we were able to gain some results on the approximate efficacy of the six InsP<sub>5</sub> isomers (four of which are presented as racemic pairs) as substrates. Using 300 nM substrate, the rate of phosphorylation of InsP<sub>5</sub> isomers relative to InsP<sub>6</sub> (InsP<sub>6</sub> = 100%) was 2-OH-InsP<sub>5</sub>, 0.5%; 1/3-OH-InsP<sub>5</sub>, 3.2%; 4/6-OH-InsP<sub>5</sub>, 3.0%, and 5-OH-InsP<sub>5</sub> showed no detectable phosphorylation over background levels. In parallel experiments, *scyllo*-inositol hexakisphosphate was phosphorylated at 4.7% of the rate of InsP<sub>6</sub>.



**Figure 1** HPLC profile of a liver InsP<sub>6</sub> assay

Three typical profiles from three assay tubes are illustrated, with the radioactivity in the InsP<sub>7</sub> region (indicated by the peak in [ $^3\text{H}$ ]InsP<sub>7</sub> elution) shown. Left-hand axis (◆), [ $^3\text{H}$ ]; right-hand axis (■), [ $^{32}\text{P}$ ]. Note the differing scales on the [ $^{32}\text{P}$ ] axes [dpm (d.p.m.)]. (A) Blank incubation (control), (B) 5  $\mu\text{l}$  of liver extract, and (C) 5  $\mu\text{l}$  of liver extract with 10 pmol InsP<sub>6</sub> added just before the assay was performed.

We explored whether other compounds present in rat tissue extracts might interfere with the assay by 'spiking' samples with known quantities of InsP<sub>6</sub>. We found significant levels of interference caused by factors present in the extracts, which varied considerably between tissues (from a 2–10-fold underestimate of the InsP<sub>6</sub> added), and we attribute this to endogenous ATP diluting our [ $\gamma$ - $^{32}\text{P}$ ]ATP, plus other unknown compounds inhibiting the enzyme. We tried a number of 'purification' protocols to separate InsP<sub>6</sub> from these interfering factors, but this only led to losses

**Table 1** *InsP<sub>6</sub>* content of tissues and fluids

Tissues were extracted and assayed as described in the Experimental section. Each assay was performed in triplicate, and results are means  $\pm$  S.E.M. For all tissues, at least one independent assay was performed on another sample, with similar results (results not shown). Note that the *InsP<sub>6</sub>* concentration for all samples except serum, plasma and urine, is an estimated intracellular concentration.

Tissue	<i>InsP<sub>6</sub></i> (pmol/mg of protein)	<i>InsP<sub>6</sub></i> concentration
Rat liver	39.8 $\pm$ 0.43	11 $\pm$ 0.12 $\mu$ M
Rat brain	194.7 $\pm$ 25.5	14.5 $\pm$ 1.9 $\mu$ M
Rat kidney	55.5 $\pm$ 7.1	10.9 $\pm$ 1.4 $\mu$ M
Rat lung	69.8 $\pm$ 12.4	15.8 $\pm$ 2.8 $\mu$ M
HeLa cells	175 $\pm$ 16.6	37.6 $\pm$ 3.5 $\mu$ M
Slime mould	3645 $\pm$ 114	352 $\pm$ 11 $\mu$ M
Calf serum	–	< 8 nM
Human erythrocytes	0.037 $\pm$ 0.0057	26 $\pm$ 4.0 nM
Human white cells	43.5 $\pm$ 6.1	–
Human serum	–	< 0.5 nM
Human plasma	–	< 0.5 nM
Human urine	–	< 5 nM

of *InsP<sub>6</sub>*, so, in the end, we decided to adopt a protocol where we performed six assays for any single tissue sample. Three are aliquots of the tissue extract, and three are identical aliquots spiked just before the assay with a known amount (usually 10 pmol) of *InsP<sub>6</sub>*. From the difference between these latter spiked samples and the unspiked samples, we can calculate the d.p.m. of *InsP<sub>7</sub>* formed from the known *InsP<sub>6</sub>*, and comparing this with a 10 pmol standard assayed at the same time quantifies the ‘quenching’ present in each individual tissue sample. This can then be used to correct the *InsP<sub>7</sub>* formed in the unquenched samples. This is a rather laborious process (as we also included blank incubated controls in all experiments), but it does allow us to be confident that each sample is quantitatively assayed.

This approach assumes that the quenching is linear with *InsP<sub>6</sub>* concentration (doing a complete standard curve within each sample would be the only rigorous way of ensuring that). Although we believe such linearity is indeed likely, some non-linearity would not seriously compromise the conclusions drawn below other than to lead to an underestimate of *InsP<sub>6</sub>* in some tissues listed in Table 1. Whenever possible, we used quantities of cell extract that contained approximately the same amount of *InsP<sub>6</sub>* as the added spike, which self-evidently helps to ensure linearity and also simplifies the quantification of the degree of quenching. We tested our extraction protocol by spiking several samples of rat tissues with [<sup>3</sup>H]*InsP<sub>6</sub>* before extraction, and found that we recovered 80–85% up to the point of adding the enzyme to the assay. We have corrected all our results for tissue extracts by assuming 80% recovery.

### *InsP<sub>6</sub>* content of rat tissues

Figure 1 shows a typical set of single HPLC profiles from an assay performed on an extract from rat liver. The combination of the triplicate samples leads to a level of 4.75 pmol in the sample, which represents 0.84 mg of wet weight of tissue (0.149 mg of protein). If we assume an 80% recovery and that there is 0.64 g water/g of tissue (see [33,34] for derivation of this latter assumption), then the complete assay leads to an estimated intracellular level of 11  $\pm$  0.12  $\mu$ M *InsP<sub>6</sub>* in the liver.

In a further set of experiments, we then assayed *InsP<sub>6</sub>* levels in a selection of rat tissues, and the results are shown in Table 1. We observe no obvious pointers to tissue-specific functions in these results, as the concentrations of *InsP<sub>6</sub>* were broadly similar. Perhaps the most relevant observation in that regard is that compar-

ing the two internal tissues in the body exposed to the highest and lowest O<sub>2</sub> levels (lung and kidney respectively), there is no large difference. If a principal function of *InsP<sub>6</sub>* *in vivo* was to act as an antioxidant [26,35], then one might expect there to be much more *InsP<sub>6</sub>* in the lung. In fact, the likelihood of *InsP<sub>6</sub>* interacting with iron ions (the reason for its antioxidant action) inside cells has been questioned in the light of the discovery that *InsP<sub>6</sub>* probably exists mostly as a penta-Mg<sup>2+</sup> salt *in vivo* [36].

### Slime moulds

Our calculated concentrations of *InsP<sub>6</sub>* in mammalian tissues are overall consistent with the indirect calculations derived from equilibrium labelling of cell cultures with [<sup>3</sup>H]inositol (e.g. [6,37]), but nevertheless we sought an independent validation of the assay. One organism for which there is already an accurate mass estimation of *InsP<sub>6</sub>* is *D. discoideum*, which has an estimated intracellular concentration of *InsP<sub>6</sub>* assayed by inorganic phosphorous determination as 594  $\mu$ M [8]. This value is significantly higher than the estimated maximum solubility of the penta-Mg<sup>2+</sup> salt of *InsP<sub>6</sub>* proposed to exist *in vivo* (approx. 50  $\mu$ M [38]), and may be explained by some form of compartmentalization in acidic vesicles (see [38] for discussion).

We therefore assayed pellets of axenal cultures of slime moulds for *InsP<sub>6</sub>*. The pellets were weighed before extraction, and the mass levels of *InsP<sub>6</sub>* measured gave an estimated concentration in the cells of 352  $\pm$  11  $\mu$ M (Table 1). The figure of 594  $\mu$ M [8] used an assumption of 0.4 g of water/g of pelleted cells, which corrects to 371  $\mu$ M if we use the assumption of 0.64 g of water as determined above. The close agreement between the two sets of data provides an independent validation of the present assay, and confirms the really remarkably high *InsP<sub>6</sub>* levels in these organisms.

### HeLa cells

We next assayed *InsP<sub>6</sub>* in confluent cultures of HeLa cells (Table 1). These have apparently higher levels of *InsP<sub>6</sub>* than rat tissues; however, the difficulty of accurately measuring cellular wet weight, plus the absence of connective tissue in a cell culture, limit the interpretability of such comparisons. Nevertheless, these experiments provided us with an opportunity to investigate the extent to which mammalian cell lines make all their own *InsP<sub>6</sub>*. *InsP<sub>6</sub>* is reported to be present in human and rat serum or plasma [17–19], so it is possible that cultured cells derive some or even all of their *InsP<sub>6</sub>* from the calf serum in the medium in which they are grown. In this respect, cell cultures could be argued to be a paradigm for whole animal requirements, as they are with inositol (see [39] and the Discussion section). So we tried to measure *InsP<sub>6</sub>* in the calf serum in which the cells were cultured, but could detect none within the limits of the assay. The arguments for these limits are detailed below when we consider human serum, and, using this logic, we estimate that there could be no more than 16 pmol of *InsP<sub>6</sub>* in the medium of each 10-cm-diameter dish of cells. Yet a dish of HeLa cells grown to confluence in that medium contained 1.952  $\pm$  0.117 nmol of *InsP<sub>6</sub>*. The same medium has been used in our laboratory to grow numerous other cell lines [e.g. HEK-293 cells (human embryonic kidney cells), COS cells and CHO cells (Chinese-hamster ovary cells)] for many passages, and so from these results we conclude that cultured mammalian cells make all their own *InsP<sub>6</sub>*.

### Human blood

The absence of detectable *InsP<sub>6</sub>* in calf serum raised the question of whether this is unique to cows, given that human and rat serum

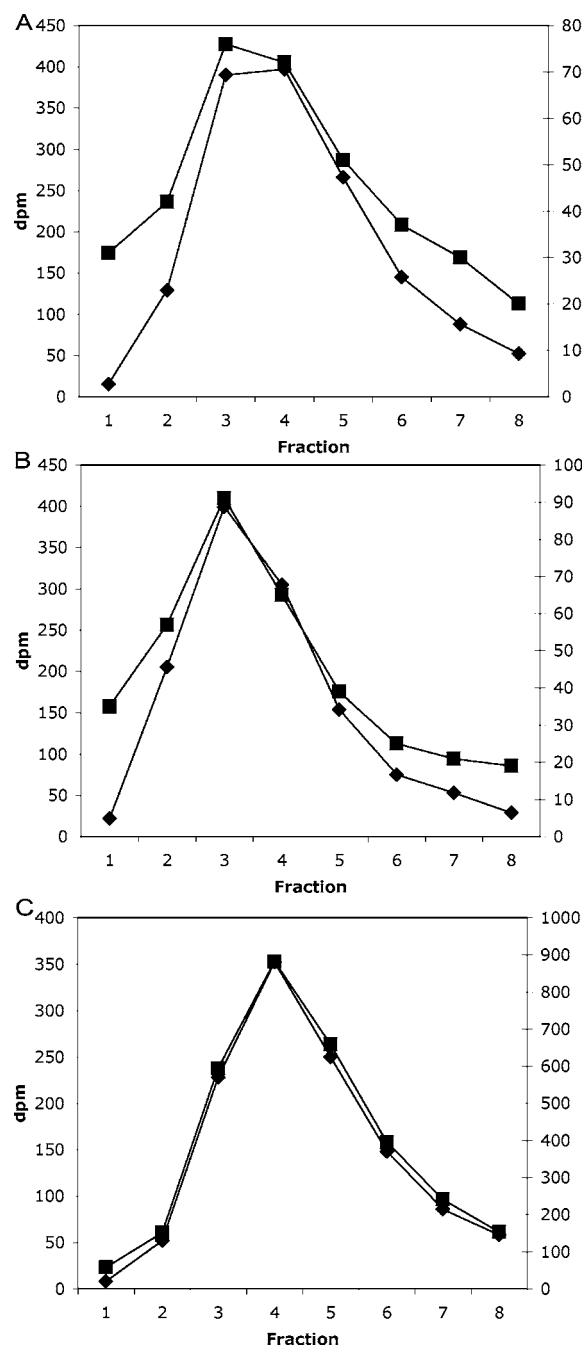
are reported to have levels of  $\text{InsP}_6$  from 30–460 nM (e.g. [19,25] and see also the Discussion section). So we tested human blood. We fractionated the blood in two ways. As serum would be a direct comparison with the calf serum above, for some samples we let the blood clot and took the supernatant (serum) remaining. For comparison with measurements of plasma  $\text{InsP}_6$ , and also to gain some information about  $\text{InsP}_6$  in some of the cells in blood, we also collected blood into citrate, and fractionated it into erythrocytes, white (buffy coat) cells and platelet-free plasma (we discarded the platelet fraction).

The results in Table 1 show that we could detect virtually no  $\text{InsP}_6$  in erythrocytes (the small amount present is probably a result of white-cell contamination), which is in itself an interesting comparison with nucleated erythrocytes of amphibians and reptiles, which make high levels of  $\text{Ins}(1,3,4,5,6)\text{P}_5$  [6,40]. For the white cell (buffy coat) fraction, we only used cellular protein as a standard, and the results suggest that, as one might expect from nucleated mammalian cells, human white cells contain  $\text{InsP}_6$  at a concentration similar to the rat tissues assayed above (Table 1).

However, in both serum and plasma, we again found no significant quantities of  $\text{InsP}_6$ . To illustrate this point, Figure 2 shows typical HPLC profiles from assays on human serum samples which were (Figure 2C) or were not (Figure 2B) 'spiked' with 2.5 pmol of  $\text{InsP}_6$  before extraction; this extra part of the protocol was introduced to ensure that there was no extraction problem with this particular type of preparation (which has no cells, but a high protein content). Similar experiments were performed on blood taken from three individuals and all gave similar results. From these profiles (Figure 2), it is difficult to be accurate as to how much  $\text{InsP}_6$  we would have been able to detect. The background in the  $^{32}\text{P}$  channel where  $[\text{H}]\text{InsP}_7$  elutes varied between experiments, in general this was lower if the  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was fresh [27], as this  $^{32}\text{P}$  background in the HPLC eluate is the ultimate limitation to the sensitivity of the assay. A further complication is that, in that background, there is frequently an apparent minor peak of  $^{32}\text{P}$  that approximately co-chromatographs with  $[\text{H}]\text{InsP}_7$ , which is present in both blank and human serum samples (Figures 2A and 2B). Nevertheless, if we suggest that 100 d.p.m. above background in triplicate samples would have shown up as a distinct and detectable  $^{32}\text{P}$  peak co-incident with  $[\text{H}]\text{InsP}_7$  in Figure 2(B), we can calculate that this would represent no more than 64 fmol of  $\text{InsP}_6$ , which leads us to suggest that the plasma sample typified by Figure 2 probably contains less than 0.5 nM  $\text{InsP}_6$ . Moreover, during plasma preparation, some damage may have occurred to white cells (or platelets) to release traces of  $\text{InsP}_6$ , and, with this in mind, we suggest that *in vivo* blood probably contains no extracellular  $\text{InsP}_6$  at all.

### Human urine

The other human fluid that has been analysed for  $\text{InsP}_6$  is urine, which is reported to contain 1–3  $\mu\text{M}$   $\text{InsP}_6$  (e.g. [20] and see the Discussion section). We therefore measured  $\text{InsP}_6$  in a urine sample from one of our blood donors, again spiking some samples before extraction as well as afterwards to confirm the recovery of  $\text{InsP}_6$ . The results revealed that there is no detectable  $\text{InsP}_6$  present. There is more 'interference' with the assay from unknown factors present in urine than in plasma or serum, which made the assay less sensitive. But the assays included 50  $\mu\text{l}$  of samples of urine spiked with 2.5 pmol  $\text{InsP}_6$  (final concentration of 50 nM) before extraction, and these showed a clear peak of  $\text{InsP}_7$  product; from a similar semi-quantitative logic to that stated above, we can set a lower limit of  $\text{InsP}_6$  detection in the samples analysed of approx. 250 fmol  $\text{InsP}_6$ , the equivalent of 5 nM  $\text{InsP}_6$  in human urine.



**Figure 2** HPLC profile of a human plasma  $\text{InsP}_6$  assay

Three typical HPLC profiles from three assay tubes are illustrated, with the radioactivity in the  $\text{InsP}_7$  region (indicated by the peak in  $[\text{H}]\text{InsP}_7$  elution) shown. Left-hand axis (◆),  $[\text{H}]$ ; right-hand axis (■),  $[\text{P}^{32}]$ . Note the differing scales on the  $[\text{P}^{32}]$  axes [dpm (d.p.m.)]. (A) Blank incubation (control), (B) extract of 100  $\mu\text{l}$  of human plasma, and (C) extract of 100  $\mu\text{l}$  of human plasma with 2.5 pmol (25 nM final concentration)  $\text{InsP}_6$  added before extraction.

### DISCUSSION

The levels of  $\text{InsP}_6$  that we have measured in mammalian tissues are of a similar order to those deduced from equilibrium labelling of cultured cells with  $[\text{H}]\text{inositol}$  (e.g. [37], and see [6] for discussion) and we can conclude that mammalian cells contain tens of  $\mu\text{M}$   $\text{InsP}_6$ . If anything, these may be underestimates compromised by a potential non-linearity of the assay when performed on tissue extracts. Moreover, these calculations assume a uniform

cytosolic localization, but obviously local concentrations may be higher. This may be most relevant in the context of well defined nuclear functions for this inositol phosphate and its more highly phosphorylated derivatives [11,41–43]. Slime moulds, which synthesize very high levels of  $\text{InsP}_6$  very quickly [8], remain an enigma only deepened by our results.

Crucially, we have shown, as far as we know, for the first time, that HeLa cells in culture (and by implication, all mammalian cell lines in perpetuity) synthesize all their own  $\text{InsP}_6$ . This depends, of course, on the cells having sufficient inositol to make  $\text{PtdIns}(4,5)\text{P}_2$  ([39] and also see the Introduction and Discussion sections), and we suggest that they probably serve as a paradigm for humans. The key to this observation is our discovery that cultured cells have no significant external source of  $\text{InsP}_6$  because there is none detectable in the calf serum in which we grow them. This was the most unexpected finding of our study, and because of its implications for human diet and health, we went on to demonstrate, as thoroughly as the assay allows, that there is also no detectable  $\text{InsP}_6$  in human serum, plasma (Figure 2) or urine.

These results have important implications for the apparent uptake of exogenous  $\text{InsP}_6$  into cells or tissues, and for the proposed extracellular actions of  $\text{InsP}_6$  in health and disease. To consider cells first, when radiolabelled  $\text{InsP}_6$  is added at micromolar concentrations to mammalian cells in culture it has been reported to be taken up, but the radiolabel is then found as lower inositol phosphates or inositol [23,44].  $\text{InsP}_6$  binds strongly to  $\text{Ca}^{2+}$ , and its solubility limit in the presence of millimolar levels of  $\text{Ca}^{2+}$  is less than  $1\ \mu\text{M}$  [36,38]. However, the (insoluble)  $\text{Ca}^{2+}$  salt of  $\text{InsP}_6$  has been found to exist naturally, for example, as a component of the extracellular coat around the parasitic cestode *Echinococcus granulosus* [45] to which it can be transported as nanometre diameter granules [46]. From these observations, it seems that the most plausible mechanism for cellular  $\text{InsP}_6$  uptake is endocytotic absorption (possibly as a  $\text{Ca}^{2+}$ - $\text{InsP}_6$  precipitate), accompanied by dephosphorylation within the cell.

Consistent with this argument are results from the classic studies of Eagle et al. [39], in which mammalian cell lines in culture were shown to require approx.  $0.3\ \mu\text{M}$  inositol for growth, and this requirement could be fully substituted for by inositol monophosphates, or, with a much lower efficacy, by  $1\ \mu\text{M}$   $\text{InsP}_6$ . In this context, we should note that adding  $\text{InsP}_6$  at much higher (up to millimolar) concentrations to cell cultures will reduce extracellular  $\text{Ca}^{2+}$  to  $1\ \mu\text{M}$  or lower [36], so any subsequent cell death caused by such high concentrations (e.g. [21,22], and see [23] for a review) would be simply explained by the very well established requirement for extracellular  $\text{Ca}^{2+}$  in order to maintain mammalian cell function [47]; in short, the cells die because  $\text{InsP}_6$  removes all the multivalent cations from the culture medium.

Our results have an impact most directly on the issue of the uptake of  $\text{InsP}_6$  from the human diet. In a number of studies, Grases et al. [17–19] have reported that adding  $\text{InsP}_6$  to the diet, or the topical application of  $\text{InsP}_6$ -containing creams [20], increases the levels of  $\text{InsP}_6$  in plasma, urine and tissues. We have not explored different dietary regimes here, but we would argue that any such difference is not likely to be crucial. Typical levels of  $\text{InsP}_6$  in urine from human subjects kept on an 'InsP<sub>6</sub>-free diet' are 2–3  $\mu\text{M}$  [20], and in plasma approx.  $0.1\ \mu\text{M}$  [19], whereas reverting to an 'InsP<sub>6</sub>-normal diet' leads to approx. 4-fold increases in these parameters. Yet our estimates of maximal levels of  $\text{InsP}_6$  in human subjects consuming a normal, carnivorous, cereal-containing diet are respectively (at least) 200 times (plasma) and 500 times (urine) lower than those reported for human subjects free of exogenous  $\text{InsP}_6$  [17–19]. These maxima are only estimates, and perhaps a more direct demonstration of the inconsistency between results published previously [17–20]

and ours lies the results for human serum in Figure 2(C) [and the corresponding results for urine (results not shown)], where spiking serum or urine with  $\text{InsP}_6$  concentrations that are still respectively 4-fold and 40-fold lower than those claimed to be found in these fluids in  $\text{InsP}_6$ -deprived individuals [17–19] gives robust signals in our assay that are very much higher than those from the unspiked samples.

For these reasons, we suggest it is our assay that differs from other studies, not the diet of the donors. Experiments feeding radioactive  $\text{InsP}_6$  to rats showed that it is dephosphorylated within the gut, and that the radiolabel is subsequently found in plasma and urine only as inositol and  $\text{InsP}$  [48], and it has been shown that microbes in the gut contribute the phytase activity responsible for this hydrolysis [49]. As the measurement of  $\text{InsP}_6$  has so far been largely performed by (Dowex) ion-exchange chromatography (e.g. [18]) or HPLC [25] of acid extracts, followed by dephosphorylation of the fractions with phytase and then determination of *myo*-inositol (with a *scyllo*-inositol standard) by MS, it is conceivable that, as also suggested by Shears [7], the presence of high concentrations of anions (including multivalent anions such as ATP) in the tissue extracts might compromise inositol phosphate separation protocols characterized in their absence.

Thus although our assay is not designed for the quantification of lower inositol phosphates, we can combine our results with the literature published previously [48,49] to suggest that  $\text{InsP}_6$  is not absorbed directly from the diet, but rather is dephosphorylated before it is taken up; note that the results of Wise and Gilbert [49] confirm that in the absence of any hydrolysis by gut flora, there is no significant  $\text{InsP}_6$  absorption. The inositol and  $\text{InsP}$  generated by dephosphorylation will of course not be without effect: inositol is synthesized *de novo* by animals (from glucose 6-phosphate via  $\text{Ins3P}$ , see [6]), but most mammalian tissues do not produce enough of it, and so inositol is a vitamin (vitamin Bh) for which a significant dietary source is  $\text{InsP}_6$  from cereals. Free levels of inositol are high in many mammalian cells (e.g. [50]), but the  $K_m$  for  $\text{PtdIns}$  synthetase is also high [51], so if raising levels of dietary  $\text{InsP}_6$  leads to increases in the availability of inositol and  $\text{InsP}$  to tissues, a mass-action effect might be expected on  $\text{PtdIns}$  synthetase, perhaps leading to increases in steady-state inositol lipid and phosphate levels.

So, if the conclusion of the present study is that we (humans) do indeed make all our  $\text{InsP}_6$  (by the route described in the Introduction), and that we derive none directly from our diet, what effect(s) will exogenous  $\text{InsP}_6$ , something claimed to be of significant medical benefit [23,24], cause? From the arguments in the preceding paragraph, increases may occur in cellular inositide levels (including  $\text{InsP}_6$ ), but this effect would be indirect, and high dietary inositol or  $\text{InsP}$  would be expected to do the same thing. Any specific and direct actions of exogenous  $\text{InsP}_6$  will be confined to its place of application. Such direct actions would stem from its ability to bind all multivalent cations with high affinity [36,38]. Binding  $\text{Fe}^{3+}$  gives  $\text{InsP}_6$  unique and potent antioxidant properties [26,35], so it is likely that dietary  $\text{InsP}_6$  will alter oxidative processes within the gut. This, and its general chelation of cations (e.g.  $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$  and  $\text{Ca}^{2+}$ ) to restrict their uptake, probably underlie the reported effects of  $\text{InsP}_6$  *in vivo* (e.g. [23,24]). These ion-binding actions of  $\text{InsP}_6$  can have undesirable consequences, either environmental, such as depleting  $\text{Fe}^{3+}$  or  $\text{Zn}^{2+}$  from some soils [52], or physiological, by  $\text{InsP}_6$  removing these same essential cations from the human diet to a degree that can be deleterious, depending on the nutritional status of the individual [53–56].

However, a major thrust of many of the arguments for the benefits of  $\text{InsP}_6$  to humans [23,24] stems from a combination of

two factors: (a) the killing of tumour-cell cultures by applying to them high extracellular levels of  $\text{InsP}_6$ , which, we argue above, is of questionable physiological relevance; and (b) an assumption that exogenous  $\text{InsP}_6$  is taken up directly to form a significant extracellular  $\text{InsP}_6$  pool, which bathes some or all our cells and which, because of its unique chemistry, can in turn influence their physiology or pathology. It is this latter assumption that the results in the present study suggest requires reconsideration.

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