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Do mammals make all their own inositol hexakisphosphate?

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Abbreviations used: The correct nomenclature for the product of InsP₆ phosphorylation by InsP₅ kinase is PPInsP₅, and we have used this nomenclature when necessary for unambiguity (e.g. when specifying isomers). However, for most of the paper we have used InsP₇ as a simpler alternative.
ABSTRACT

A highly specific and sensitive mass assay for inositol hexakisphosphate (Ins\(_6\)) was characterised. This centres around phosphorylating Ins\(_6\) with \([^{32}P]\)-ATP using a recombinant Ins\(_6\) kinase from \(Giardia\ lambia\), followed by HPLC of the \([^{32}P]\)-labelled products with an internal \([^3H]\)-Ins\(_7\) standard. This assay was used to quantify Ins\(_6\) levels in a variety of biological samples. Concentrations of Ins\(_6\) in rat tissues varied from 10-20 \(\mu\)M (assuming 64% wet weight of tissue is cytosol water), whereas using the same assumption axenic \(Dictyostelium\ discoideum\) cells contained 352 ± 11 \(\mu\)M Ins\(_6\). HeLa cells were seeded at low density and grown to confluence, at which point they contained Ins\(_6\) levels per mg protein similar to the rat tissues. This amounted to 1.952 ± 0.117 nmoles Ins\(_6\) per culture dish, despite the cells being grown in serum shown to contain no detectable (less than 20 pmoles per dish) Ins\(_6\). These data demonstrate that mammalian cells synthesise all their own Ins\(_6\). Human blood was analysed, and although the white cell fraction contained Ins\(_6\) at a concentration comparable with other tissues, in serum and platelet-free plasma no Ins\(_6\) was detected (<1 nM Ins\(_6\)). Human urine was also examined, and also contained no detectable (<5 nM) Ins\(_6\). These data suggest that dietary studies purporting to measure Ins\(_6\) at micromolar concentrations in human plasma or urine may not have been quantifying this inositol phosphate. Therefore, claims that administrating Ins\(_6\) in the diet or applying it topically can produce health benefits by increasing extracellular Ins\(_6\) levels may require reassessment.
INTRODUCTION

Ins$P_6$ 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)$P_3$ as a second messenger [3] led to the realisation that Ins$P_6$ 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 synthesise Ins$P_6$ was controversial [6], but recent studies have led to a likely consensus. Unlike slime moulds [8] and plants [9], which can synthesise Ins$P_6$ 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)$P_3$ is formed by phospholipase C action on PtdIns(4,5)$P_2$, and the Ins(1,4,5)$P_3$ is then is phosphorylated up to Ins$P_6$. The synthesis of Ins(1,3,4,5,6)$P_5$ from Ins(1,4,5)$P_3$ is most likely to be mediated by a two-step conversion catalysed by a single enzyme, the inositol polyphosphate ‘multikinase’ Ipk2 [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)$P_3$ to Ins(1,3,4,5,6)$P_5$. The alternative route from Ins(1,4,5)$P_3$ to Ins(1,3,4,5,6)$P_5$ via Ins(1,3,4,5)$P_4$. Ins(1,3,4)$P_3$ and Ins(1,3,4,6)$P_4$ is probably quantitatively minor, at least in fibroblasts, as suggested by the data of Leyman et al [14] who showed that mouse embryonic fibroblasts devoid of any Ins(1,4,5)$P_3$ 3-kinase isoforms synthesised Ins$P_6$ at a rate indistinguishable from wild-type fibroblasts; in contrast, fibroblasts derived from Ipk2 knock-out mice show a greatly compromised Ins$P_6$ synthesis [13]. The final step of Ins$P_6$ synthesis (as in plants and slime moulds [8, 9]) is the 2-phosphorylation of Ins(1,3,4,5,6)$P_5$ by Ins$P_5$ 2-kinase [11, 15], an enzyme also essential to viable mammalian development [16].

However, although we now know that mammalian cells can synthesise Ins$P_6$, how they probably do so, and that it is essential that they do, the contribution of exogenous Ins$P_6$ to mammalian Ins$P_6$ homoeostasis is not so clear. A number of studies have reported increases in Ins$P_6$ levels in tissue and body fluids resulting from the supply of exogenous Ins$P_6$ [17-20], and other work has suggested that Ins$P_6$ 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 Ins$P_6$ (e.g. [23, 24] and many of these are predicated on the idea that exogenous Ins$P_6$ can directly and significantly influence both extra- and intracellular Ins$P_6$ levels in the body.

We have here designed and characterised an unambiguous mass assay for Ins$P_6$. 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 data provide some new insights into Ins$P_6$ levels in a number of tissues and address whether mammalian cells can make all their own Ins$P_6$. Our results also cast doubts on the idea that Ins$P_6$ is directly taken up into human tissues and cells.

EXPERIMENTAL

Reagents
All chemicals and biochemicals used were of Analar grade unless specified otherwise. [\textsuperscript{3}H]-InsP\textsubscript{6} was from PerkinElmer. A [\textsuperscript{3}H]-InsP\textsubscript{5} standard (probably 5-PP-InsP\textsubscript{5} - see Results) was prepared by incubating [\textsuperscript{3}H]-InsP\textsubscript{6} with Giardia InsP\textsubscript{6} kinase under the normal assay conditions (below) such that approximately 50\% of the InsP\textsubscript{6} was phosphorylated. Sources of InsP\textsubscript{5} isomers and scyllo-inositol hexakisphosphate were as described in ref [26].

**InsP\textsubscript{6} kinase**

This is a recombinant enzyme from *Giardia lambia*. The sequence of *Giardia* InsP\textsubscript{6} kinase (Genbank accession #AY227443) was amplified from *Giardia* genomic DNA (the gene contains no introns), and then cloned into the Hex-his bacterial vector pET43A, which fuses the solubility-enhancing bacterial protein NusA to the amino terminus of the fusion protein. The enzyme was purified by talon bead absorption and imidazole elution - for full details see Letcher et al [27]. As discussed there, there is no obvious reason why a recombinant mammalian InsP\textsubscript{6} kinase should not be substituted for the *Giardia* enzyme in this assay, although the high affinity and specificity of the *Giardia* InsP\textsubscript{6} kinase (see Results) does make it particularly suitable in this context.

**Extraction protocol**

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

**InsP\textsubscript{6} Mass Assay**

Samples of tissue extract were incubated in a final volume of 300 \( \mu l \) containing the following: 30 \( \mu l \) 10x assay buffer (500 mM HEPES/NaOH pH 7.5, 1 M KCl, 50 mM MgCl\(_2\), 10 mM EGTA, 5 mg/ml BSA); 6\( \mu l \) 600 \( \mu M \) \( \beta \)-mercaptoethanol; 10 \( \mu l \) \( \gamma \)-[\textsuperscript{32}P]-ATP (10 \( \mu Ci \)). After 60 mins at 37\°C, 45 \( \mu l \) 0.1 M Glucose and 6 \( \mu l \) hexokinase (Sigma, 20 units) were added, and the incubation was continued at 37\°C for another 90 mins to convert all the remaining radioactivity to \([\textsuperscript{32}P]\)-Glucose-6-phosphate. The reaction was stopped by addition of 0.5 ml 0.4 M ammonium formate/0.1 M formic acid, and the samples were loaded onto 0.5 ml Dowex Formate in a BioRad Econo Column (with a disc of Whatman Filter paper No 1 on top of the Dowex resin) [29]. Tubes were rinsed onto the column with 4 ml of 0.4 M ammonium formate/0.1 M formic acid.
acid, and the columns were washed with 10 mls 0.8 M ammonium formate/0.1 M formic acid to remove any remaining $[^{32}P]$-Glucose-6-phosphate. The higher inositol phosphates were eluted with 2x1 ml 2.5 M ammonium formate/0.1M formic acid. They were spiked with 600-2000 dpm of $[^{3}H]$-Ins$P_7$, and loaded onto a Whatman Patisil 10 SAX WCS analytical hplc column, 4.6 mm x 250 mm (Cat No 4226-001), or a similar size 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; Solution B, 1.2 M (NH$_4$)$_2$HPO$_4$ adjusted to pH 3.8 with phosphoric acid. Profile gives % of solution B: 0 min 0%; 5 min 0%; 30 min 78%; 75 min 78%; 76 min 100%; 81 min 100%; 82 min 0%; 102 min 0%.

One ml fractions were usually collected between 40 and 75 minutes (Ins$P_7$ elutes at about 60 mins), and were counted in a scintillation counter after adding 10 ml Ultima-Flo AP ammonium phosphate-tolerant scintillation fluid. The $[^{3}H]$ and $[^{32}P]$ windows were calibrated by 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 8 M urea by sonication (Decon ultrasonic bath) and vortexing. The majority of material dissolves, but in samples with a high fat content some fat remains in suspension and may interfere with the assay (this did not happen in any sample analysed in this report). Samples were adjusted to 6 M urea, and assayed for protein by the BioRad protein assay, based on Bradford [30], using BSA in 6 M urea as a standard.

**Blood fractionation**

Blood was taken from unfasted human volunteers who denied having taken aspirin in the preceding 48h, mixed with acid citrate dextrose (97 mM Na citrate, 78 mM citric acid, 110mM D-glucose) and centrifuged for 20 min at 200 $g$ (all centrifugations were at 4°C). The supernatant from this centrifugation (platelet-rich plasma) was further centrifuged at 2,500 $g$ 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 resuspension buffer (10 mM HEPES pH 7.4, 10 mM glucose, 1 mM MgSO$_4$, 145mM NaCl, 5 mM KCl), and then centrifuged (2,500 $g$, 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 resuspension buffer, and re-centrifuged (2,500 $g$ 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 (2,500 $g$ 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 hours, then centrifuged (3,000 g) for 20 minutes, 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 a.m., three hours 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**

**Characterisation of the enzyme and the assay**

Using [3H]InsP₆ we established that the *Giardia* kinase has a Km of approximately 60 nM (not shown). We used this enzyme to make our own [3H]-InsP₇ standard for HPLC. Because the *Giardia* gene is closely related to InsP₆ kinases from other eukaryotes we assume that its product is 5-[PP]InsP₅ (see [31] for discussions), though we have not established its structure. Draskovic et al [32] have shown that the mammalian InsP₅ 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]InsP₅ synthesised from InsP₆ by a mammalian InsP₅ kinase (a gift from Dr S.B. Shears) to the *Giardia* enzyme for several hours, but detected no formation of any InsP₈, which suggests that *Giardia* InsP₆ kinase is a 5-kinase too, and that extra phosphorylation of 5-[PP]InsP₅ is not a complicating factor in the InsP₅ assay.

In the context of this assay the crucial point is that we always include a [3H]InsP₇ standard made by the *Giardia* enzyme in every HPLC run, and collect and count 1 min samples individually for both isotopes. This leads to a precise co-chromatography of the [3H]-labelled standard and the [32P]-labelled product formed from InsP₆ in the cell extract (e.g. Fig 1); any sample in which this co-chromatography was not absolutely precise was discarded (except for some blanks, where low levels of radioactivity mean that the correlation is less evident – see below). Note that in the Figures the left hand axis (diamonds) is always the [3H]-labelled InsP₇ standard, and the right hand axis (squares), whose scale varies greatly between Figures (which serves to illustrate precise co-chromatography of the two isotopes), is the [32P] counts for the same fractions.

We have discussed elsewhere [27], and discounted, the possibility of InsP₅ in extracts interfering significantly with the assay. In brief: InsP₅s are poor substrates for the *Giardia* enzyme (see below); any PPIInsP₄ products formed elute well before InsP₇ under our hplc protocol; and any tri-phosphorylated InsP₃ [32] would be formed in minute amounts during our 60 min incubations, and also would not precisely co-elute with 5-[PP]InsP₅. High backgrounds in the region of the HPLC profile where PPIInsP₄ elutes prevented us from extensive quantitative analysis of InsP₅s as substrates for the *Giardia* InsP₆ kinase. However, in one set of experiments with a fresh batch of [32P]-γ-ATP (and
consequently lower background) we were able to gain some data on the approximate efficacy of the six InsP<sub>5</sub> isomers (four of which are presented as racemic pairs) as substrates. Using 300nM 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%; 5-OH-InsP<sub>5</sub> showed no detectable phosphorylation over background. In parallel experiments scyllo-inositol hexakisphosphate was phosphorylated at 4.7% the rate of InsP<sub>6</sub>.

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 2 to 10-fold underestimate of the InsP<sub>6</sub> added), and we attribute this to endogenous ATP diluting our [<sup>32</sup>P]-γ-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 of InsP<sub>6</sub>, so in the end we decided to adopt a protocol where we did 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 picomoles) of InsP<sub>6</sub>. From the difference between these latter spiked samples and the unspiked we can calculate the dpm of InsP<sub>7</sub> formed from the known InsP<sub>6</sub>, and comparing this with a 10 picomole 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 include 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). While 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 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 enzyme to the assay. We have corrected all our data for tissue extracts by assuming 80% recovery.

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

Fig 1 shows a typical set of single HPLC profiles from an assay performed on an extract of rat liver. The combination of the triplicate samples leads to a level of 4.75 pmoles in the sample which represents 0.84 mg wet weight of tissue (0.149 mg of protein). If we assume an 80% recovery and that there is 0.64g water per gram of tissue (see [33, 34] for derivation of this latter assumption), then the complete assay leads to an estimated intracellular level of 11 ± 0.12 µM InsP<sub>6</sub> in 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 data, as the concentrations of InsP<sub>6</sub> were broadly similar.
Perhaps the most relevant observation in that regard is that comparing the two internal tissues in the body exposed to the highest and lowest O₂ levels (lung and kidney respectively), there is no large difference. If a principle function of \( \text{InsP}_6 \) \textit{in vivo} were to act as an anti-oxidant [26, 35] then one might expect there to be much more \( \text{InsP}_6 \) in lung. In fact the likelihood of \( \text{InsP}_6 \) interacting with iron ions (the reason for its anti-oxidant action) inside cells has been questioned in the light of the discovery that \( \text{InsP}_6 \) probably exists mostly as a penta-Mg\(^{2+}\) salt \textit{in vivo} [36].

**Slime moulds**

Our calculated concentrations of \( \text{InsP}_6 \) in mammalian tissues are overall consistent with the indirect calculations derived from equilibrium labelling of cell cultures with \(^{[3]H}\) 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 \( \text{InsP}_6 \) is \textit{Dictyostelium discoideum}, which has an estimated intracellular concentration of \( \text{InsP}_6 \) assayed by inorganic phosphorous determination as 594 \( \mu \text{M} \) [8]. This value is significantly higher than the estimated maximum solubility of the penta-Mg\(^{2+}\) salt of \( \text{InsP}_6 \) proposed to exist \textit{in vivo} (about 50 \( \mu \text{M} \) [38]), and may be explained by some form of compartmentalisation in acidic vesicles (see [38] for discussion).

We therefore assayed pellets of axenal cultures of slime moulds for \( \text{InsP}_6 \). The pellets were weighed before extraction, and the mass levels of \( \text{InsP}_6 \) measured gave an estimated concentration in the cells of 352 ± 11 \( \mu \text{M} \) (Table 1). The figure of 594 \( \mu \text{M} \) [8] used an assumption of 0.40g water per gram of pelleted cells, which corrects to 371 \( \mu \text{M} \) if we use 0.64g as here. The close agreement between the two sets of data provides an independent validation of the present assay, and confirms the really remarkably high \( \text{InsP}_6 \) levels in these organisms.

**HeLa cells**

We next assayed \( \text{InsP}_6 \) in confluent cultures of HeLa cells (Table 1). These have apparently higher levels of \( \text{InsP}_6 \) than the 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 \( \text{InsP}_6 \). \( \text{InsP}_6 \) 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 \( \text{InsP}_6 \) 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 ref [39] and Discussion). So we tried to measure the \( \text{InsP}_6 \) 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 picomoles of \( \text{InsP}_6 \) in the medium of each 10 cm dish of cells. Yet a dish of HeLa cells grown to confluence in that medium contained 1.952 ± 0.117 nmoles \( \text{InsP}_6 \). The same medium has been used in our laboratory
to grow numerous other cell lines (e.g. HEK-293, COS, CHO) for many passages, and so from these data we conclude that cultured mammalian cells make all their own InsP₆.

**Human blood**

The absence of detectable InsP₆ 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 InsP₆ from 30-460 nM (e.g. [19, 25] and see Discussion). 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 InsP₆, and also to gain some information about InsP₆ 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 data in Table 1 show that we could detect virtually no InsP₆ in erythrocytes (the small amount there is probably due to white cell contamination), which is in itself an interesting comparison with nucleated erythrocytes of amphibians and reptiles which make high levels of Ins(1,3,4,5,6)P₅ [6, 40]. For the white cell (‘buffy coat’) fraction we only used cellular protein as a standard, and the data suggest that, as one might expect from nucleated mammalian cells, human white cells have InsP₆ at a concentration similar to the rat tissues assayed above (Table 1).

However, in both serum and plasma, we again found no significant InsP₆. To illustrate this point, Fig 2 shows typical HPLC profiles from assays on human serum samples which were (Fig 2c) or were not (Fig 2b) ‘spiked’ with 2.5 pmoles of InsP₆ 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 (Fig 2), it is difficult to be accurate as to how much InsP₆ we would have been able to detect. The background in the [³²P] channel where [³H]-InsP₇ elutes varied between experiments, in general being lower if the [³²P]-γ-ATP was fresh [27] – this [³²P] background in the HPLC eluate is the ultimate limitation to the assay’s sensitivity. A further complication is that in that background there is frequently an apparent minor peak of [³²P] that approximately co-chromatographs with the [³H]-InsP₇, which is present in both blank and human serum samples (Figs 2a and 2b). Nevertheless, if we suggest that 100 dpm above background in triplicate samples would have shown up as a distinct and detectable [³²P] peak co-incident with [³H]-InsP₇ in Fig 2b, we can calculate that this would represent no more than 64 fmole of InsP₆, which leads us to suggest that the plasma sample typified by Fig 3 probably contains less than 0.5 nM InsP₆. Moreover, during plasma preparation some damage may have occurred to white cells (or platelets) to release traces of InsP₆, and with this in mind we suggest that in vivo blood probably contains no extracellular InsP₆ at all.

**Human urine**
The other human fluid that has been analysed for InsP₆ is urine, which is reported to contain 1-3 µM InsP₆ (e.g. [20]) and see Discussion). We therefore measured InsP₆ in a urine sample from one of our blood donors, again spiking some samples before extraction as well as afterwards to confirm recovery of InsP₆. The data revealed that there is no detectable InsP₆ 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 µl samples of urine spiked with 2.5 pmol of InsP₆ (final concentration 50 nM) before extraction, and these showed a clear peak of InsP₆ product; from a similar semi-quantitative logic to that above we can set a lower limit of InsP₆ detection in the samples analysed of about 250 fmoles InsP₆, the equivalent of 5 nM InsP₆ in human urine.

DISCUSSION

The levels of InsP₆ that we have measured in mammalian tissues are of a similar order to those deduced from equilibrium labelling of cultured cells with [³H]-inositol (e.g. [37] and see [6] for discussion) and we can conclude that mammalian cells contain tens of µM InsP₆. 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 localisation, 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 synthesise very high levels of InsP₆ very quickly [8], remain an enigma only deepened by our data.

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) synthesise all their own InsP₆. This depends, of course, on them having sufficient inositol to make PtdIns(4,5)P₂ ([39] and see Introduction and discussion below), 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 InsP₆ 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 InsP₆ in human serum, plasma (Fig 2) or urine.

These data have important implications for the apparent uptake of exogenous InsP₆ into cells or tissues, and for the proposed extracellular actions of InsP₆ in health and disease. To consider cells first, when radiolabelled InsP₆ 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]. InsP₆ binds strongly to Ca²⁺, and its solubility limit in the presence of millimolar levels of Ca²⁺ is less than 1 µM [36, 38]. However, the (insoluble) Ca²⁺ salt of InsP₆ 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 nm diameter granules [46]. From these observations it seems that the most plausible mechanism for cellular InsP₆ uptake is endocytotic absorption (possibly as a Ca²⁺-InsP₆ precipitate), accompanied by dephosphorylation within the cell.
Consistent with this argument are data from the classic studies of Eagle et al. [39], in which mammalian cell lines in culture were shown to require about 0.3 µM inositol for growth, and this requirement could be fully substituted for by inositol monophosphates, or, with a much lower efficacy, by 1 µM InsP₆. In this context we should note that adding InsP₆ at very much higher (up to millimolar) concentrations to cell cultures will reduce extracellular Ca²⁺ to 1 µM or lower [36], so any subsequent cell death caused by such high concentrations (e.g. [21, 22] and see [23] for review) would be simply explained by the very well established requirement for extracellular Ca²⁺ to maintain mammalian cell function [47]; in short, the cells die because InsP₆ removes all the polyvalent cations from the culture medium.

Our data impact most directly on the issue of the uptake of InsP₆ from the human diet. In a number of studies, Grases and colleagues have reported that adding InsP₆ to the diet [17-19], or the topical application of InsP₆-containing creams [20], increases levels of ‘InsP₆’ 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 ‘InsP₆’ in urine from human subjects kept on an ‘InsP₆-free diet’ are 2-3 µM [20], and in plasma around 0.1 µM [19], while reverting to an ‘InsP₆ normal’ diet leads to approximately 4-fold increases in these parameters. Yet our estimates of maximal levels of InsP₆ 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 InsP₆ [17-19]. These maxima are only estimates, and perhaps a more direct demonstration of the inconsistency between published data and ours lies the data for human serum in Fig 2C (and corresponding data for urine, not shown), where spiking serum or urine with InsP₆ concentrations that are still respectively four-fold and forty-fold lower than those claimed to be found in these fluids in InsP₆-deprived individuals [17-19] give 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 InsP₆ to rats showed that it is rapidly dephosphorylated within the gut, and that the radiolabel is subsequently found in plasma and urine only as inositol and InsP [48] - it has been shown that microbes in the gut contribute the phytase activity responsible for this hydrolysis [49]. As the measurement of ‘InsP₆’ 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 mass spectrometry, it is conceivable that, as also suggested by Shears [7], the presence of high concentrations of anions (including polyvalent anions such as ATP) in the tissue extracts might compromise inositol phosphate separation protocols characterised in their absence.

Thus although our assay is not designed for the quantification of lower inositol phosphates, we can combine our data with the published literature to suggest that InsP₆ is not absorbed directly from the diet, but rather is dephosphorylated before it is taken up; note that the data of Wise and Gilbert [49] confirm that in the absence of any hydrolysis by gut flora, there is no significant InsP₆ absorption. The inositol and InsP generated by dephosphorylation will of course not be without effect: inositol is synthesised de novo by animals (from glucose 6-phosphate via Ins3P, see [6]), but most mammalian tissues do
not make 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 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 PtdIns synthetase, perhaps leading to increases in steady-state inositol lipid and phosphate levels.

So, if the conclusion of our 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 with high affinity all polyvalent cations [36, 38]. Binding \( \text{Fe}^{3+} \) gives \( \text{InsP}_6 \) unique and potent anti-oxidant 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. \( \text{Fe}^{3+}, \text{Zn}^{2+}, \text{Ca}^{2+} \)) to restrict their uptake, probably underlie \( \text{InsP}_6 \)'s reported effects \textit{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 data in our study suggest requires reconsideration.

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| Tissue                | InsP₆ (pmoles/mg protein) | InsP₆ concentration |
|-----------------------|---------------------------|---------------------|
| Rat Liver             | 39.8 ± 0.43               | 11 ± 0.12 µM        |
| Rat Brain             | 194.7 ± 25.5              | 14.5 ± 1.9 µM       |
| Rat Kidney            | 55.5 ± 7.1                | 10.9 ± 1.4 µM       |
| Rat Lung              | 69.8 ± 12.4               | 15.8 ± 2.8 µM       |
| HeLa cells            | 175 ± 16.6                | 37.6 ± 3.5 µM       |
| Slime Mould           | 3645 ± 114                | 352 ± 11 µM         |
| Calf Serum            | ----                      | < 8 nM              |
| Human erythrocytes    | 0.037 ± 0.0057            | 26 ± 4.0 nM         |
| Human White cells     | 43.5 ± 6.1                | ----                |
| Human Serum           | ----                      | < 0.5 nM            |
| Human Plasma          | ----                      | < 0.5 nM            |
| Human Urine           | ----                      | < 5 nM              |

Tissues were extracted and assayed as described in the text. Each assay was done in triplicate, and results are expressed as ± S.E.M. For all tissues at least one independent assay was performed on another sample, with similar results. Note that the InsP₆ concentration for all samples except serum, plasma and urine, is an estimated intracellular concentration (see text for details).
FIGURE LEGENDS

Figure 1
Hplc profile of a liver InsP₆ assay. Three typical assay tubes are illustrated, with the radioactivity in the InsP₇ region (as located by the peak of [³H]-InsP₇ elution) shown. Diamonds and left axis, [³H]; Squares and right axis, [³²P]. Note differing scales on the [³²P] axes.
A, blank incubation; B, 5µl liver extract; C, 5µl liver extract with 10 pmoles InsP₆ added just before assay.

Figure 2
Hplc profile of a human plasma InsP₆ assay. Three typical assay tubes are illustrated, with the radioactivity in the InsP₇ region (as found by the peak of [³H]-InsP₇ elution) shown. Diamonds and left axis, [³H]; Squares and right axis, [³²P]. Note differing scales on the [³²P] axes.
A, blank incubation; B, Extract of 100µl human plasma; C, Extract of 100µl Human plasma with 2.5 pmoles (25 nM final concentration) InsP₆ added before extraction.
