A novel metal–dye detection system permits picomolar-range h.p.l.c. analysis of inositol polyphosphates from non-radioactively labelled cell or tissue specimens

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A novel complexometric dye- and transition-metal-based post-column detection system for polyanions, called ‘metal–dye detection’ has been developed. This technique, combined with a new h.p.l.c. separation protocol, permits a direct highly-isomer-selective determination of bis- to poly-phosphorylated non-radioactively labelled compounds in the picomolar range, a sensitivity hitherto unknown for these substances. The application of the technique in the quantitative microanalysis of inositol polyphosphates from milligram amounts of cells or tissue specimens is described. The technique promises to answer hitherto unresolved questions about the role of inositol phosphates, especially those in intact tissues, which are not readily amenable to analysis by radioisotopic techniques.

INTRODUCTION

Most data published on agonist-induced changes in cellular inositol phosphates are based on radioisotopic techniques. Cells or small tissue samples are mostly incubated with *myo*-[³H]inositol in order to obtain labelled inositol phosphates. In many tissue specimens, an equilibrium of labelling is barely achieved in the time period in which functional integrity is maintained, a fact precluding estimates of concentrations of these metabolites. The alternative technique, namely [³²P]P_i labelling, shows a strong interference of labelled nucleotides and other phosphorylated compounds with inositol phosphates upon h.p.l.c. analysis [1]. Furthermore, the specific radioactivities may be rapidly altered upon agonist stimulation [2]. In spite of these problems, isotopic techniques have, in many cases, permitted the evaluation of agonist-induced changes in inositol phosphates and inositol phospholipids [3–5]. However, in general they provide only relative estimates of fluxes; with very few exceptions [6,7], absolute concentration data are still missing.

Knowledge of absolute concentration changes in inositol phosphates in cells, as well as access to the microanalysis of inositol phosphates in specimens obtained from intact tissues, would certainly answer many hitherto unresolved questions about the role of this signalling system *in vivo* [8].

A recently published non-radiometric h.p.l.c. technique [7], employing dephosphorylation in an enzyme-loaded post-column reactor and subsequent P_i analysis, required relatively large amounts of tissue and, furthermore, showed a strong interference of incompletely dephosphorylated higher InsP_x (inositol polyphosphate) isomers ($x \geq 4$) with the molybdate complex; peaks were suppressed or even negative, precluding a reliable

quantification. In the search for an alternative, more sensitive, detection method, I succeeded with a novel dye-based ternary-complexometric technique not requiring dephosphorylation. Its principle is based on the finding that tervalent transition-metal ions bind with very high affinity to both the cation-specific dye 4-(2-pyridylazo)resorcinol (PAR) [9] and polyanions like inositol polyphosphates. In this ‘two-ligand one-metal’ system the dye functions as a reporter substance, indicating optically the presence of competing ligands. This intriguingly simple optical technique, which may be termed ‘metal–dye detection’ (m.d.d.) promises to fulfil most of the requirements for a non-radiometric microanalysis of InsP_x. Furthermore, a novel strongly acidic h.p.l.c. elution protocol effected an isomer selectivity of inositol phosphates with more than three phosphate groups, better than that achieved up until now.

MATERIALS AND METHODS

Analytical-grade HCl was from Riedel de Haen (Seelze, Germany); triethanolamine (lot E7), Dowex AG1 X2 resin (200–400 mesh, Cl[−] form) and activated charcoal (Norit A) were from Serva (Heidelberg, Germany). Norit A was further treated by boiling for 2 h in 3 M-HCl, washing with water to neutrality and drying at 120 °C. Mono Q anion-exchange columns (HR 5/5 and HR 5/20) and Q-Sepharose (fast flow) were from Pharmacia, Uppsala, Sweden. Transition metals (99.9% pure, as trichloride hexahydrates) were from Janssen (Beerse, Belgium) and Aldrich (Milwaukee, WI, U.S.A.). Ins1P, Ins4P, Ins(1,4)P₂, Ins(4,5)P₂, Ins(1,4,5)P₃, Ins(2,4,5)P₃, Ins(1,4,5,6)P₄, Ins(1,3,4,5,6)P₅, Fru(1,6)P₂, 3-phosphoglycerate and phosphoenolpyruvate were from Boeh-

Abbreviations used: PAR, 4-(2-pyridylazo)resorcinol; GroPIns(4,5)P₂, glycerophosphoinositol 4,5-bisphosphate; InsP, InsP₂, InsP₃, InsP₄, InsP₅, and InsP₆, *myo*-inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphate, with positional isomerism of phosphoesters as indicated in parentheses; InsP_x, inositol polyphosphate(s); 2,3-BPG, D-glycerate 2,3-bisphosphate; m.d.d., metal–dye detection; f.m.i., focused-microwave irradiated.

ringer, Mannheim, Germany. All other phosphorylated compounds, polycarbonic acids, sodium phytate and PAR were from Sigma (St. Louis, MO, U.S.A.). $\text{Ins}(1,3,4,5)P_4$ was from Amersham-Buchler, Braunschweig, Germany. $\text{Ins}(1,2,3,4,5)P_5$ was isolated from partly hydrolysed phytic acid by Dowex 1 chromatography. Conditions of hydrolysis and of chromatography were essentially as described in [10]. Two peaks of $\text{Ins}P_5$ were obtained, of which the first smaller one ('IP5A' according to [10]) was identified by n.m.r. as pure $\text{Ins}(1,2,3,4,5)P_5$.

N.m.r. investigation of isolated $\text{Ins}P_x$ isomers

$\text{Ins}P_x$ preparatively isolated were structurally assigned by proton, ^{13}C and ^{31}P n.m.r. The instrument used, and the techniques employed, were described in [11]. Detailed data from these investigations are available from me on request.

High-performance anion-exchange chromatography and on-line detection

A Pharmacia h.p.l.c. system (two P-3500 pumps, LCC-500 controller with gradient-mixing valve, UV-M monitor, and ACT-100 autosampler) or, alternatively, the inert h.p.l.c. system from LKB (two 2150 pumps, 2152 LC-controller and 2151 monitor) were used. All wetted parts of the h.p.l.c. system were made from Teflon, titanium or plastics, and glass columns of the HR 5 type from Pharmacia were packed with Mono 1 (10 μm -particle-size, monodisperse low-capacity anion-exchange beads). A filter unit containing 8 mm-diameter cellulose discs and a hydrophobic 1 μm -pore-size filter (Schleicher und Schüll) protected the column. The weak eluent, A, contained 0.2 mM-HCl and varied concentrations of transition metal, normally 9 or 18 μM ; the strong eluent, B, contained 0.4 M-HCl and normally 14 or 28 μM -transition metal. Upward-concave gradients were employed for elution. The dye solution, C, contained 100–500 μM -PAR (added from a 20 mM stock solution in methanol) and 1.3 M-triethanolamine, adjusted to pH 8.4 with HCl. All solutions were Millipore-filtered (1 μm pore size) and stored at room temperature. Reagent C was routinely added to the column eluent by means of a h.p.l.c. pump, but a Gilson-type Minipuls 2 peristaltic pump was also adequate. A filter unit as described above was installed after this pump. The volume ratio of reagent C to eluent A/B was 1:2 (v/v). A mixing T-junction and a knitted coil made from 0.5 mm-internal-diameter Teflon tubing of 1 m length (200 μl volume) were optimal for mixing without peak broadening. Detection was routinely at 546 nm, for maximal detection sensitivity at 520 nm. The monitor was auto-zeroed after starting each chromatogram, and the inverted signal was recorded, baseline-subtracted and integrated by a C-R5A Chromatopac from Shimadzu, Düsseldorf, Germany.

For simultaneous liquid-scintillation counting, a fraction of the eluent was withdrawn via a mixing T-junction before the addition of reagent C by a peristaltic pump, and, after adjusting the delay to that of the monitor signal, peaks were pooled by hand after this signal. Here the HCl eluent has the advantage of causing no chemiluminescence.

Preparation of samples

Extraction. Cell suspensions were directly extracted by

adding ice-cold HClO_4 to a final concentration of 0.5 M to the suspending buffer (which should be free of sulphate and low in phosphate, EGTA and other polyvalent anions). Tissue specimens were either freeze-clamped or focused-microwave-irradiated (f.m.i.). Frozen specimens were powdered in a liquid- N_2 -cooled steel-ball mill (Braun-Melsungen). The frozen powder, or f.m.i. specimens cooled on ice, were homogenized in 2 ml of ice-cold 0.5 M- HClO_4 in a small Ultra-Turrax homogenizer for 20 s. Internal standard [normally $\text{Ins}(1,2,3,4,5)P_5$] was added immediately after HClO_4 addition. After removal of the HClO_4 precipitate formed after 20 min on ice, the 5000 g supernatant was adjusted to a pH of maximally 5 by adding KOH, and the KClO_4 precipitate formed after 20 min on ice was removed by centrifugation. To facilitate the pH adjustment, 25 mM acetic acid was normally added together with HClO_4 .

Charcoal treatment of samples. Except for control samples, nucleotides were removed by charcoal treatment. A 20% (w/v) suspension of acid-treated Norit A (see above) was prepared in 0.1 M-NaCl/50 mM-sodium acetate, pH 4.0. Samples, processed as described above, were freeze-dried, redissolved in 1 ml of water at ambient temperature, the pH was re-adjusted to below 5 with KOH or HCl, and these samples, transferred to Eppendorf tubes, were then twice charcoal treated. A 50 μl portion of the Norit A suspension was added per 25 mg wet weight of tissue or cells, and the suspension was thoroughly vortexed-mixed five times over a 15 min period. After a 3 min centrifugation in an Eppendorf centrifuge at maximal speed, supernatants were once more treated with the same amount of charcoal in a second tube. The two charcoal pellets were consecutively re-extracted with 1 ml of 0.1 M-NaCl and the resulting re-extract was combined with the sample.

Solid-phase extraction. If samples contained a lot of salt, as in case of cells suspended in larger volumes of buffer and after charcoal treatment, this treatment was performed. The sample was diluted with water to > 15 ml and applied on to a disposable column containing 0.5 ml of Q-Sepharose (adjusted to be in the Cl^- form). After washing twice with 4 ml of 2.5 mM-HCl, the inositol polyphosphates were eluted with 2×2.5 ml of 0.6 M-HCl. This eluate was immediately frozen in polypropylene tubes as a thin layer and freeze-dried to remove the HCl. Dried samples were dissolved in 2.2 ml of 5 mM-sodium acetate, pH 5.0. Care was taken to wet the whole surface of the polypropylene tubes several times. Samples were usually stored frozen at this stage.

Immediately before h.p.l.c. analysis, the samples were thawed, centrifuged to remove dust, and 2.1 ml was transferred into 2.3 ml Eppendorf tubes. These tubes were covered with Parafilm (gloves had to be used; otherwise phosphatase contamination was observed). Covered tubes, inserted into 5 ml scintillation vials, were mounted in the ACT 100 autosampler. Sample injection was with a 2 ml sample loop.

RESULTS AND DISCUSSION

Realization of the m.d.d. technique

In Fig. 1 the new post-column detection principle for inositol polyphosphates and its realization with an h.p.l.c.

Fig. 1. Realization of a h.p.l.c. system with metal-dye detection

(a) The principle of detection; (b) the build-up of the h.p.l.c. system employed. M stands for the trivalent transition metals (Y, La, Nd, Gd, Ho, Lu).

system are depicted. The two essential components of the detection system are a trivalent transition metal, which was included in the elution buffers here, and the cation-specific dye, PAR, which was continuously added to the column eluate. This dye shows little absorbance at 520 nm in the absence of cations, but transition-metal-dye complexes very strongly absorb at this wavelength with specific absorbances up to $60\,000\text{ M}^{-1}$ [9]. When complexed with metal to a certain extent, such as occurs in the mixture of PAR with the eluate, the dye can 'sense' the presence of ligands in the eluate competing for the metal. In this case it will bind less cation in favour of the competitor, and, in parallel, its absorbance at 520 nm will decrease. In order to facilitate an integration of the resulting chromatograms containing negative peaks, the detector signal was routinely inverted. If the dye/eluant mixture has a pH above 7.5, phosphorylated compounds become very strong polycation-complexing agents that are sensitively detectable by this technique.

The acidic elution system that was employed (0.2 mM to 0.4 M-HCl) prevents a strong interaction or precipitation of the metal with phosphorylated compounds while they are separated on the column. Furthermore, under these acidic elution conditions, a drastic increase of isomer selectivity was obtained for inositol polyphosphates containing more than three phosphate groups. Styrol-based anion-exchange h.p.l.c. matrices were found to be sufficiently acid-resistant to maintain full performance for more than 1000 runs, and inert h.p.l.c. systems are today of technical standard. Acid-labile phosphorylated compounds such as nucleoside polyphosphates, polymetaphosphates and glyceryl phosphodiester compounds [e.g. $\text{GroPIns}(4,5)\text{P}_2$] were not hydrolysed by these elution conditions.

The dye, PAR, was post-column-mixed with the eluent

together with a monobasic buffer. This buffer is needed to bring the eluate/dye mixture to alkalinity for detection (see above). Triethanolamine, adjusted to pH 8.4 with HCl, proved to be ideally suited on the basis of its pK value (7.7) and its cost. Its buffer capacity was adjusted in such a way as to adjust the eluent pH to distinctly above 7. This significantly increases the stabilities of the inositol phosphate-metal complexes and the PAR-metal complex, and thus the sensitivity. Furthermore, it minimizes the pH-dependent change in the absorbance of PAR.

Compatibility of m.d.d. with different elution systems

Besides the above-described elution system, I also investigated whether some of the 'milder' elution protocols employed in many studies (e.g. [5,13,14]) are compatible with the novel detection principle. The univalent anions chloride, formate and acetate were employed as eluting anions without problems, whereas sulphate and, especially, phosphate form stronger complexes with the trivalent metals, thus precluding their use as eluents. As counter-ions, organic univalent cations of large ionic radius are best suited because they do not, or only weakly, bind to PAR. Cations which significantly bind to PAR decrease the detection sensitivity. Tetramethylammonium, Tris, triethanolamine, imidazole, but also ammonium, were found to be usable. Therefore ammonium formate buffers, which are frequently employed to separate inositol phosphates, can be used unless they contain phosphoric acid. When the pH of elution buffers is below 6, the trivalent transition metal can normally be added to these buffers. At higher pH values, precipitation with polyanions and the formation of insoluble hydroxo complexes can occur. In these instances, both the transition metal and the dye can be premixed with a slightly acidic detection reagent which is added to the column eluate.

Optimization of the sensitivity of m.d.d.

Among the series of trivalent transition metals tested as 'indicator cations' (Y, La, Nd, Gd, Ho, and Lu), Ho and Y, both with crystal ionic radii of 0.09 nm (0.9 Å), led to the highest detection sensitivity (Fig. 2a). Y was superior to Ho, however, in causing less pH-dependent baseline drift and showing a higher sensitivity for InsP_2 detection. The linear detection range (see Fig. 2b) could be adjusted between 5 and 1000 pmol and between 0.05 and 10 nmol by varying the type of metal (cf. Fig. 2a) and its final concentration (between 5 and 50 μM). At a low concentration of transition metal, the fraction associated with PAR decreased. As this resulted from the approach towards the K_a of the metal-PAR complex, it could be opposed via the Mass Action effect by increasing the concentration of PAR. Final concentrations of PAR above 200 μM , however, proved to be unfavourable, because of the high basal absorbance of PAR plus univalent-cation-complexed PAR and because of a slow precipitation reaction occurring at higher concentrations.

The highest specific absorbance that was obtained at 520 nm (the wavelength of maximal metal-specific absorbance of metal-PAR complexes) exceeded $300\,000\text{ M}^{-1}$ (for InsP_6); at 546 nm it still amounted to $150\,000\text{ M}^{-1}$, which is about 10 times the specific u.v. absorbance of ATP. Photometric measurements under similar conditions indicated that the K_a values for binding to more

Fig. 2. Properties of the m.d.d. system

(a) Dependence of detection sensitivity by m.d.d. on the ionic radius of the transition metal used. In each case, 18 μM and 28 μM of metal in eluent A and B respectively and 500 μM -PAR in reagent C were used. As a test mixture of inositol polyphosphates, partly chemically hydrolysed phytic acid [9] was used. An amount corresponding to 10 nmol of total InsP_x was injected on to a 5 cm column of Mono Q, and each of the inositol polyphosphate peaks separated (one InsP_2 , four InsP_3 s, six InsP_4 s, four InsP_5 s, InsP_6) were individually integrated. Peak areas obtained with La were set to 1. Areas obtained for other transition metals were calculated relative to these data. Relative values within groups of InsP_x thus obtained varied and were averaged. The mean value of the five averages obtained for InsP_2 – InsP_6 is shown by $\bullet$. Individual relative sensitivities obtained for InsP_2 and InsP_6 are depicted by $\circ$ and $\square$ respectively. The sensitivities for individual peaks eluted between InsP_2 and InsP_6 were always between these values. A strong dependence of relative detection sensitivity on the crystal ionic radius (values were taken from [12]) is evident, the optimum lying at a radius of 0.09 nm (0.9 Å). (b) Linearity of detection of InsP_x by m.d.d. The substances specified in the Figure were injected on to a 5 cm column of Mono Q and the peak areas obtained were plotted against the picomolar quantities injected. Reagent C contained 100 mM-PAR, and eluents A and B contained 9 and 14 μM - YCl_3 respectively. As demonstrated here for ATP, nucleotides showed a slight parabolic deviation from linearity at lower concentrations, apparently caused by incomplete complexation with the transition metal.

than monophosphorylated inositols are far below 100 nm (at pH 8) for all tested transition metals and that the stoichiometry of metal binding generally parallels the number of phosphate groups. These extremely high affinities are the rationale for the excellent linearity of detection in the picomolar range (Fig. 2b) and the extreme detection sensitivity (see Fig. 3).

Interferences with inositol phosphate detection

Whereas between pH 3.7 and 7 nucleoside tri- and di-phosphates are eluted close to InsP_3 and InsP_2 respectively [1,5,7,13], with the HCl system all nucleoside triphosphates were eluted before InsP_3 . ATP, which is present in large amounts in extracts, was eluted even ahead of InsP_2 (Fig. 3). Thus the interference coming from nucleotides, which are also detected by this method, is kept to a minimum (cf. Fig. 4). Nevertheless, there was still interference by rarer nucleotides and oligonucleotides. These could be almost completely eliminated by

the charcoal treatment described in the Materials and methods section. When attempting to optimize this sample-processing step, I discovered that even acid-treated commercial charcoal has a significant potential to bind highly phosphorylated inositol phosphates in anionic-strength-dependent manner, i.e. it behaves like an anion-exchanger. This property can lead to a complete loss of inositol polyphosphates if they are carrier-free. The problem was overcome by two simple improvements. One was to diminish the 'anion-exchange capacity' of the charcoal by boiling each batch in acid, and by limiting the amount of charcoal added to a sample. The second, much more effective, improvement was to perform the charcoal treatment at high ionic strength and acidic pH. High ionic strength was achieved by performing a HClO_4 extraction/ KClO_4 precipitation instead of trichloroacetic acid extraction/ether extraction or HClO_4 extraction/Freon/tri-*N*-octylamine extraction [14]. When KClO_4 is precipitated at 0 °C, more than

Fig. 3. H.p.l.c.-m.d.d. analysis of a standard mixture of nucleotides and InsP_x .

Separation was performed on a 15 cm $\times$ 0.5 cm Mono Q column. Eluent A and B contained 9 and 14 μM - YCl_3 respectively, and reagent C contained 200 μM -PAR. The flow rate was 1.2 ml/min for A/B, and 0.6 ml/min for C. The gradient applied is depicted. The upper monitor tracing (a) was obtained by m.d.d. at 546 nm (5 mm-path-length cell), the lower one (b) by u.v. detection at an identical absorbance setting. Note the low degree of peak broadening by m.d.d. and the significantly higher detection sensitivity of m.d.d. for nucleotide polyphosphates. The components of the nucleotide mixture are given in the lower tracing. The mixture of InsP_x was from partly hydrolysed InsP_6 [10]. An equivalent of 10 nmol of InsP_x was injected. Assignments are in part based on pure standards available. For further assignments, the InsP_6 hydrolysate was preparatively fractionated by HCl gradient elution from a Dowex-1 column [10]. Neutralized pooled peak fractions were subjected to n.m.r. spectroscopy [11] for the identification of InsP_x isomers present and re-analysed on the h.p.l.c. system for peak positions. Assignments of isomers to individual peaks thus possible are indicated by an asterisk (*): 1, $\text{P}_i + \text{InsP}$; 2, $\text{Ins}(1,2)\text{P}_2^* + \text{Ins}(1,6)\text{P}_2^*$; 3, PP_i ; 4, unidentified; 5, $\text{Ins}(1,3,5)\text{P}_3^* + \text{Ins}(2,4,6)\text{P}_3^*$; 6, $\text{Ins}(1,3,4)\text{P}_3^*$; 7, $\text{Ins}(1,2,3)\text{P}_3^* + \text{Ins}(1,2,6)\text{P}_3^* + \text{Ins}(1,4,5)\text{P}_3^*$ preceding $\text{Ins}(2,4,5)\text{P}_3^*$; 8, $\text{Ins}(1,5,6)\text{P}_3^*$; 9, $\text{Ins}(4,5,6)\text{P}_3^*$; 10, $\text{Ins}(1,2,3,5)\text{P}_4^* + \text{Ins}(1,2,4,6)\text{P}_4^*$; 11, $\text{Ins}(1,2,3,4)\text{P}_4^* + \text{Ins}(1,3,4,6)\text{P}_4^*$; 12, $\text{Ins}(1,3,4,5)\text{P}_4^*$; 13, $\text{Ins}(1,2,5,6)\text{P}_4^*$; 14, $\text{Ins}(2,4,5,6)\text{P}_4^*$; 15, $\text{Ins}(1,4,5,6)\text{P}_4^*$; 16, $\text{Ins}(1,2,3,4,6)\text{P}_5^*$; 17, $\text{Ins}(1,2,3,4,5)\text{P}_5^*$; 18, $\text{Ins}(1,2,4,5,6)\text{P}_5^*$; 19, $\text{Ins}(1,3,4,5,6)\text{P}_5^*$; 20, InsP_6 ; a, AMP + CMP + NAD; b, cyclic AMP; c, GMP; d, NADH; e, UMP; f, NADP; g, ADP + ADP-ribose; h, ATP + CTP; i, GDP; k, IDP; l, GTP; m, UDP; n, ITP; o, UTP. Elution volumes of bisphosphates in the 23–33 ml range (not all included in this chromatogram) were (in ml): $\text{Ins}(1,3)\text{P}_2^*$, 23.28; $\text{Ins}(1,6)\text{P}_2^*$, 23.45; $\text{Ins}(1,4)\text{P}_2$, 23.95; $\text{Ins}(1,5)\text{P}_2^*$, 23.95; $\text{Ins}(1,2)\text{P}_2^*$, 23.97; sedoheptulose-1,7-bisphosphate, 24.00; glucose 1,6-bisphosphate, 24.07; $\text{Ins}(4,5)\text{P}_2$, 24.34; $\text{Ins}(2,4)\text{P}_2$, 24.35; fructose 1,6-bisphosphate 25.33; PP_i , 27.87; 2,3-BPG, 32.29.

0.1 M- ClO_4^- remains soluble. This unprecipitated ClO_4^- rendered a sufficient amount of anions in each extract. The subsequent concentration step further increased the ionic strength of the samples before charcoal treatment. The second factor preventing adsorption to the charcoal, the acidic pH, was adjusted by addition of KOH to the HClO_4 . Furthermore, a co-precipitation of inositol polyphosphate-bivalent metal complexes with the KClO_4 was effectively prevented by the acid pH. As deduced from the recovery of $\text{Ins}(1,2,3,4,5)\text{P}_5$ added to samples as an internal standard, a greater-than-90% recovery was routinely achieved by these improvements.

By the final solid-phase extraction, cations, Cl^- , organic acids, and the remaining ClO_4^- were effectively removed from the samples, with the valuable consequence of extremely stable retention times. Adsorption to an anion-exchanger, with consecutive desorption with HCl,

was developed. By using Q-Sepharose as the anion-exchanger, all inositol phosphates were eluted by about half the concentration of HCl which was necessary for Dowex 1 resins. The concentration of HCl eluting also InsP_6 (0.6 M) was low enough to prevent hydrolysis or acid migration and to allow its removal by freeze-drying from a thin film.

In order to ascertain the absence of interfering nucleotides after charcoal treatment, control samples were routinely analysed by u.v. detection before the addition to the dye reagent.

Finally, I tested how other polyanions present in cell extracts interfere with the inositol phosphates in the HCl elution system. Phosphoenolpyruvate, phosphoglycerate and 6-phosphogluconate are eluted shortly after mono-phosphates. Among all the di- and poly-carbonic acids present in cells or added in buffers (e.g. EGTA or

Fig. 4. H.p.l.c.-m.d.d. analysis of InsP_x from living cells

The column length and elution protocol was as for Fig. 3, except that the final part of the gradient (from 60% B) was steepened. Absorbance was measured at 546 nm (5 mm-path-length cell). (a) Analysis of HClO_4 extracts from normoxaemic (i) and anoxaemic (ii) avian blood. Heparinized whole blood obtained from a domestic goose was equilibrated in a tonometer at 40 °C against a wet gas phase containing 5% CO_2 /75% (or 95%) N_2 and 20 (or 0%) O_2 respectively. After 2 h, a sample was HClO_4 treated as described in the text. Samples corresponding to 30 μl of blood were injected without charcoal treatment. Upon hypoxia (upper trace), a dramatic decrease of ATP, UTP (and GTP) and corresponding increase in AMP (not detected) and P_i occurs. Simultaneously, mainly two isomers of InsP_3 , namely $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,6)\text{P}_4$ [or $\text{Ins}(1,2,4,5)\text{P}_4$; Fig. 3] increase significantly, whereas apparently no isomer of InsP_3 (and InsP_2) exhibits a strong alteration. (b) Analysis of InsP_x of platelets unstimulated or 2 min after thrombin stimulation. Human platelets were isolated from buffy coats obtained from a local blood transfusion centre by differential centrifugation. They were finally suspended at a concentration of $2 \times 10^9/\text{ml}$ in a buffer containing 135 mM-NaCl, 10 mM-LiCl, 5 mM-KCl, 1 mM- MgCl_2 , 1 mM- CaCl_2 , 5 mM- NaHCO_3 , 5 mM-glucose and 10 mM-Hepes, pH 7.4. After a 30 min incubation at 37 °C, platelets were stirred magnetically and stimulated by adding thrombin to 1 unit/ml. Portions (2.5 ml) of the suspension (corresponding to about 30 mg wet wt. of platelets) were HClO_4 -extracted, charcoal-treated and solid-phase-extracted as described in the Materials and methods section. In the unstimulated cells (lower tracing, i), no $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$ or InsP_4 , but small amounts of InsP_2 , $\text{Ins}(1,3,5)\text{P}_3$ and of $\text{Ins}(1,3,4,5,6)\text{P}_5$ were detectable. Upon thrombin stimulation, there was a rapid increase of $\text{Ins}(1,3)\text{P}_2$, $\text{Ins}(3,4)\text{P}_2$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,3,4)\text{P}_3$, and $\text{Ins}(1,4,5)\text{P}_3$ and of some $\text{Ins}(1,3,4,5)\text{P}_4$ (not shown), as has been well established by isotopic techniques [6]. When the platelets started to aggregate and secrete (which occurred after about 60 s of stirring) a significant rise of several other isomers of InsP_4 and of $\text{Ins}(1,3,4,5,6)\text{P}_5$ occurred (upper unbroken tracing, iii). The chromatogram shown in the broken line (ii) was from a standard containing 1 nmol of ATP, 1 nmol of $\text{Ins}(1,4)\text{P}_2$, 1 nmol of 2,3-BPG, 500 pmol of $\text{Ins}(1,4,5)\text{P}_3$, 500 pmol of $\text{Ins}(1,3,4,5)\text{P}_4$, and 500 pmol of $\text{Ins}(1,4,5,6)\text{P}_4$.

EDTA), only the most acidic one, oxalate, is eluted significantly later than monophosphates. Monocarboxylic acids and also some of the dicarboxylic acids are removed by the solid-phase extraction (see above). The bisphosphorylated sugars glucose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate (and GTP, if not removed by charcoal treatment) interfere with isomers of InsP_2 , whereas fructose 1,6-bisphosphate is eluted distinctly later. However, using column lengths of more than 15 cm and an isocratic phase in the elution program leads to full separation of these components. PP_i , 2,3-BPG, and a component tentatively assigned to phosphoribosyl pyrophosphate, are well separated between InsP_2 and

InsP_3 . On a 15 cm column, InsP_3 is separated into five, InsP_4 into seven groups of isomers and InsP_5 into all four individual isomers (Fig. 3). When column lengths of 25–30 cm were employed, the separation of isomers of InsP_2 and InsP_3 is further improved. Assignments of InsP_x isomers to the peaks in Fig. 3 are in part based on standards available and in part on n.m.r. analysis of InsP_x fractions isolates from partly hydrolysed InsP_6 [10]. As at present the number of inositol phosphate standards available is still much less than the number of isomers theoretically possible (39, not considering enantiomorphs), it is no surprise that more positive signals are detected in charcoal-treated tissue samples than can be

assigned (see Fig. 4*b*). In order to rule out compounds other than orthophosphoric esters (e.g. polysulphated glycans), control samples were extensively treated with alkaline phosphatase ('molecular biology' grade), which leads to a complete disappearance of inositol phosphates.

Applications of the technique

By this novel technique, it has become possible to directly quantify InsP_x isomers with excellent isomer selectivity for $x > 2$, and, simultaneously, phosphorylated substances known to interfere with InsP_x metabolism from every kind of tissue. The minimal specimen wet weight is about 10 mg. For a combination of absolute quantification and measurement of fluxes of incorporated radioactive label, the column effluent can be divided into two lines, one for m.d.d. and the other for liquid-scintillation counting (see Fig. 1). In Fig. 4 two applications are shown: in (*a*), avian blood kept normoxaemic and anoxaemic respectively was analysed without charcoal treatment; in (*b*) the InsP_x isomers generated in thrombin-stimulated platelets are shown. Besides these applications in living-tissue analyses the m.d.d. technique might prove useful in fields of related analytical interest, e.g. in the picomolar detection of polyanionic water pollutants, e.g. polyphosphates or other cation chelators. Furthermore, rapid column separation protocols combined with m.d.d. in many cases could replace the expensive and laborious radioisotopic techniques exclusively employed up until now to assay InsP_x kinases and InsP_x phosphatases.

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