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Note

Serial separation of inositol phosphates including pentakis- and hexakisphosphates on small anion-exchange columns[a]

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Stepwise elution of inositol phosphates InsP, $InsP_2$, $InsP_3$ and $InsP_4$ with solutions containing increasing concentrations of ammonium formate in dilute formic acid was described by other workers [1–4]. A version allowing, in addition, for satisfactory separation of $InsP_5$ and $InsP_6$ has repeatedly been requested in the literature [5] and would appear to be useful for phosphorylation and dephosphorylation studies, preparative purposes and description of responses provoked within the cellular inositol phosphates upon physical and chemical challenges, in particular agonist–receptor interactions at the cell surface [6–8].

EXPERIMENTAL

Chemicals, resin and labelled standards

All chemicals used were of analytical grade. The resin, Dowex 1-X8 p.A. (200–400 mesh) (Serva Feinbiochemica, Heidelberg, F.R.G.), was suspended in 10 volumes of 3 *M* hydrochloric acid for a few hours. Then, *ca* 85% of the resin was allowed to settle and the remainder was removed by aspiration. After water washes the resin was treated with 3 *M* ammonium formate until free of chloride. [2-^{3}H]Inositol and ^{3}H-labelled standards for Ins(1)P, Ins(1,4)P_2, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were obtained from The Radiochemical Centre (Amersham, U.K.). [^{3}H]Ins(1,2,4,5,6)P_5, [^{32}P]$InsP_6$ and [^{32}P]Ins(1,3,4,5,6)P_5 [9] were kindly donated by L. R. Stephens, AFRC Institute of Animal Physiology (Babraham, Cambridge, U.K.). and Hionic-Fluor was purchased from Packard (Downers

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Grove, IL, U.S.A.). Glass microfiber paper GF/A was purchased from Whatman International (Maidstone, U.K.).

Cell preparation, labelling and extraction

Ehrlich ascites tumour cells proliferating in the abdominal cavity of host mice were labelled by intraperitoneal injection of 300 μCi of [2-^{3}H]myoinositol (10–20 Ci/mmol). After 18 h the cells were harvested at 37°C into a mammalian buffer, also used for subsequent washes before experimentation [10,11].

Cell suspension volumes of 500 μl containing 20 μl of cells were transferred to ice-cooled Eppendorf tubes containing 700 μl of trichloroacetic acid (final concentration 7.5%). The cells were repeatedly mixed during 20 min at 4°C for complete extraction of cellular inositol phosphates [1]. After centrifugation, 1-ml portions of clear supernatant were transferred to glass vials and equilibrated with 8 ml of water-saturated diethyl ether in three cycles in order to remove trichloroacetic acid. The diethyl ether-washed cell extract was mixed with 4 ml of 7 8 m*M* sodium tetraborate–12.5 m*M* sodium EDTA. The final pH was 8.0. Before chromatography the samples were stored at −20°C.

Anion-exchange column chromatography

The chromatographic column was a 50 mm × 5 mm I.D. glass tube with a reservoir. The resin was deposited on a circular filter punched out from GF/A glass filter "paper". Care from the glass-blower to provide a reasonably abrupt, syringe-like transition to the outlet below will ensure that this filter is positioned stably. On top of the 45-mm-high column another glass filter circle, preferably of 5 5 mm diameter was accurately arranged such that ideal geometry was reasonably approached, this point being essential for subsequent separations. Excess resin on the glass wall above the upper filter could easily be swept off with water and removed. With the outlet connected to a coiled 35 cm × 1 mm I.D. silicone rubber tubing an easily regulated and increased flow-rate was at hand. The columns were used only once. Ten to twenty columns were handled simultaneously.

Thawed samples were briefly shaken and then loaded onto water-equilibrated columns taking care to avoid splashing to areas not cleaned during the subsequent washing out of the considerable amounts of labelled inositol present in the extracts, water volumes of 8, 6 and 2.5 ml were used. The flow-rate was throughout regulated to about 0.2 ml/min. The elution protocol is presented in Table I.

Eluents 1–5 were added stepwise by hand as 3-ml volumes and eluents 6–8 as 4-ml volumes. These volumes were collected in plastic scintillation vials and mixed with 16 ml of Hionic-Fluor as scintillation counting fluid. With eluents 1–3 a clear, stable fluid phase was not obtained unless 0.3 ml of 6 *M* ammonium formate solution had been added to each vial. Counting efficiency, tested with [^{3}H] inositol as internal standard, was insignificantly different for eluents 2–8, but for eluent 1 a 5% surplus was noticed.

TABLE I

PROTOCOL FOR ELUTION OF CELLULAR INOSITOL PHOSPHATES

Related details are given in the Experimental section and in the Discussion

Eluent No	Composition	Total volume
1	0 06 *M* $HCOONH_4$–0 005 *M* $Na_2B_4O_7$	9 ml for Gro-PI
2	0 15 *M* $HCOONH_4$–0 050 *M* HCOOH	9 ml for InsP
3	0 30 *M* $HCOONH_4$–0 100 *M* HCOOH	15 ml for $InsP_2$
4	0 65 *M* $HCOONH_4$–0 100 *M* HCOOH	15 ml for $InsP_3$
5	0 80 *M* $HCOONH_4$–0 100 *M* HCOOH	15 ml for $InsP_4$
6	0 85 *M* $HCOONH_4$–0 100 *M* HCOOH	44 ml for $InsP_5$
7	1 20 *M* $HCOONH_4$–0 100 *M* HCOOH	24 ml for $InsP_6$
8	2 00 *M* $HCOONH_4$–0 100 *M* HCOOH	12 ml

RESULTS

The elution profile obtained for inositol phosphates from [^{3}H]myoinositol-labelled cells is shown in Fig. 1.

To determine the reproducibility of the separation procedure, seven columns

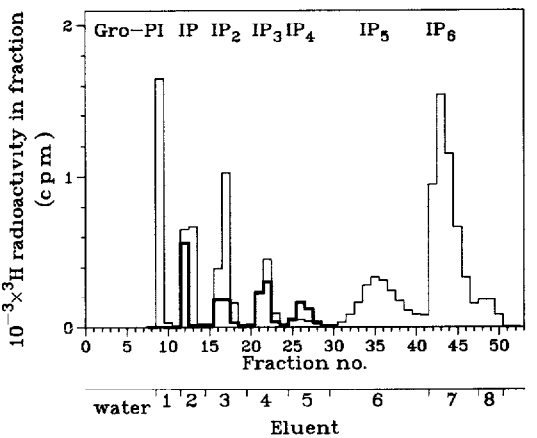

Fig 1. Separation of labelled cellular inositol phosphates on columns (45 mm × 5 mm I D) of Dowex 1-X8 anion-exchange resin in the formate form Abbreviations Gro-PI = glycerophosphoinositol, Ins(1)P = myoinositol monophosphate with ester bond at carbon atom No 1, analogous interpretations for $Ins(1,4)P_2$ etc Two columns were run in parallel Thin line elution profile for [^{3}H]myoinositol-labelled cellular compounds extracted from Ehrlich ascites tumour cells Notice that the final 2 5 ml of water (fraction 8) were found to be virtually free of labelled inositol Thick line elution profile obtained when commercial ^{3}H-labelled Ins(1)P, $Ins(1,4)P_2$, $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ standards had been added to an application volume containing extracted material from unlabelled cells Recovery of standards 90–100%

were loaded, each with 5 ml from a pool worked up from surplus cell extract. The coefficient of variation was 7% for glycerophosphoinositol (Gro-PI), 1% for InsP, $InsP_2$ and $InsP_3$, 6% for $InsP_4$, 2% for $InsP_5$ and 3% for $InsP_6$.

Fig. 2A shows that the elution profile of standard [^{32}P]Ins(1,3,4,5,6)P_5 is indistinguishable from the profile of ^{3}H-labelled cellular material present in the elution fractions containing that standard. This is consistent with the recognized predominance of the Ins(1,3,4,5,6)P_5 isomer over other $InsP_5$ isomers in mammalian cells [7,12].

Fig 2B demonstrates that the ^{32}P-labelled standard $InsP_6$ co-eluted with the ^{3}H-labelled cellular material, designated $InsP_6$, while being adequately separated from $InsP_5$

Fig. 3 shows that a ^{3}H-labelled standard for the Ins(1,2,4,5,6)P_5 isomer had a slightly shorter retention time than the Ins(1,3,4,5,6)P_5 isomer.

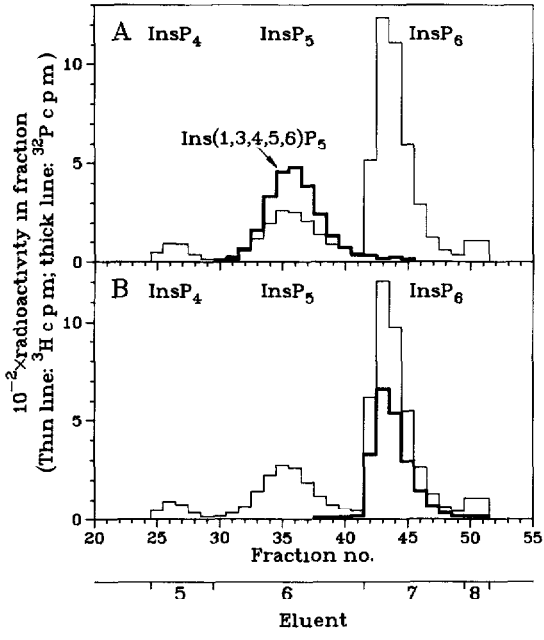

Fig 2 ^{32}P-Labelled standard Ins(1,3,4,5,6)P_5 and standard $InsP_6$ were added separately to extracts of [^{3}H]myoinositol-labelled cells before chromatography. (A) ^{32}P-Labelled Ins(1,3,4,5,6)P_5 (thick line) co-chromatographed with the cellular ^{3}H-labelled material (thin line) designated as $InsP_5$. (B) ^{32}P-Labelled $InsP_6$ (thick line) co-chromatographed with the cellular ^{3}H-labelled material (thin line) obtained with eluent 7 and designated as $InsP_6$. Radioactivities were registered by means of a preset ^{3}H/^{32}P liquid scintillation program and appropriately corrected. For both panels, $n = 2$ Recovery of added ^{32}P-labelled standards was between 94 and 100%

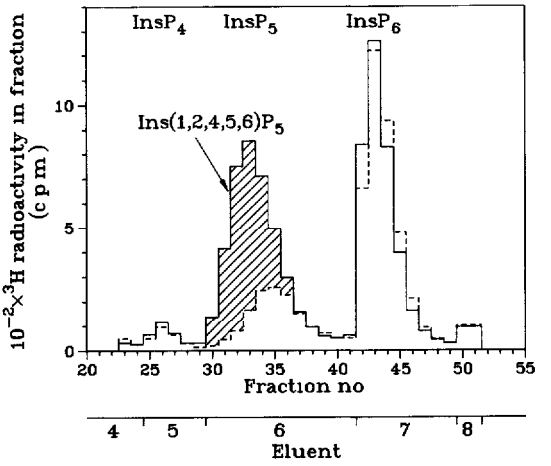

Fig 3 [^{3}H]Ins(1,2,4,5,6)P_5 standard was added to one of two 5-ml application volumes both pipetted from pooled surplus volumes derived from [^{3}H]myoinositol-labelled Ehrlich cells The elution profile (solid line) was obtained for the application volume enriched with the [^{3}H]Ins(1,2,4,5,6)P_5 standard The form of the hatched area shows that the added standard peaked slightly before cellular total $InsP_5$ radioactivity Recovery of the standard (n = 2) was 86 and 84%.

DISCUSSION

Current procedures [1–3] for anion-exchange chromatographic separation of inositol phosphates on small columns according to the number of phosphorylated hydroxyl groups have been extended to include $InsP_5$ and $InsP_6$ in serial elution. More sophisticated anion-exchange chromatographic techniques devised by Cosgrove [13], as well as the existing high-performance liquid chromatographic methods [3,7,12,14] would allow for the same, and would in addition serve to resolve the positional isomers. Thus, the ease with which many simultaneous runs are carried out by simple unexpensive means has to justify the work presented.

In this context, equally good separation (unpublished results) was lately found to result from (a) a shift to AG 1-X8 resin, 200–400 mesh, formate form, as received from Bio-Rad (Richmond, CA, U.S.A.), (b) use of a 26 mm × 6 mm resin column demarcated above and below by a GF/A filter circle (d=6.5 mm) and (c) an increase in flow-rate from 0.2 to *ca.* 0.27 ml/min through all steps involved, such that less than 10 h are needed for the application of the cell extract, washing out of labelled free inositol from the column and the serial elution of the inositol phosphates. For elution of $InsP_5$ twelve 5-ml volumes of eluent 6 are used. $InsP_6$ is eluted to 99% with 12 ml of a 1.50 M ammonium formate–0.100 M formic acid eluent.

The addition of an $InsP_6$ hydrolysate as "carrier" material [4] to the 5-ml volumes applied to the columns did not significantly influence the recovery of

cellular inositol phosphates. The general safeguard offered by appropriate addition of some $InsP_6$ hydrolysate is, nevertheless, considered in current work.

$InsP_6$ presents a particular case in that isomers for this phosphoric myoinositol ester are not existing. It is, therefore, of some interest to consider the four possible isomers of $InsP_5$ as contaminants to $InsP_6$. With reference to Fig. 2A, and by assuming equal molar specific activities, the contamination of $InsP_6$ by Ins(1,3,4,5,6)P_5 did not exceed 1% of the $InsP_6$ eluted Fig. 3 confirms a similar conclusion for any Ins(1,2,4,5,6)P_5 present. Further, referring to Cosgrove's observation [13] concerning myoinositol "that the pentaphosphate with the unsubstituted axial hydroxyl group is the most difficult to elute from an ion-exchange resin", also the two isomers of Ins$(P)_5$ other than Ins(1,3,4,5,6)P_5 and Ins (1,2,4,5,6)P_5 should only insignificantly contaminate Ins$(P)_6$ when working with mammalian cells.

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