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Preparative separation of *myo*-inositol bis- and trisphosphate isomers by anion-exchange chromatography

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ABSTRACT

Of the ion-chromatographically distinct isomers, 11 of the 12 *myo*-inositol trisphosphates (IP_3) and all of the 9 *myo*-inositol bisphosphates (IP_2) have been found in a chemical hydrolysate of phytic acid. The isomers were fractionated by anion-exchange chromatography by a method that is suitable for the purification of mg amounts of the IP_2 and IP_3 isomers.

INTRODUCTION

Interest in *myo*-inositol phosphates is focused mainly at present on their intracellular activity as second messengers[1]. However, the extracellular occurrence and action of these substances are also gaining increasing attention[2–5]. Several h.p.l.c. methods for the fractionation of mixtures of IP_x isomers* have been described, most of which are based on anion-exchange chromatography[8–11] as were the original column chromatographic methods[12–14]. Some ion-pair chromatographic approaches have also been made[15,16].

The purpose of the work now reported was to produce IP_x isomers as reference compounds for analytical systems. IP_2 and IP_3 isomers were obtained from phytic acid by hydrolysis and separated from P_i, IP_1, and IP_4–IP_6 by conventional methods. The IP_2 and IP_3 isomers were then fractionated on an anion-exchange column.

EXPERIMENTAL

The Dowex resin was obtained from Sigma and the Aminex A-27 resin from Bio-Rad. The latter was converted into the Cl^- from before use.

Sodium phytate was hydrolysed for 5 days at pH 4 and 80°; 50% of the total phytate phosphorus was released. The yields of IP_2 and IP_3 were each ~15%.

* Abbreviations: P_i, inorganic phosphate; IP_1–IP_6, *myo*-inositol mono-, bis-, tris-, tetrakis-, pentakis-, and hexakis-phosphate, respectively. The positional isomers of *myo*-inositol phosphates are numbered in accordance with the IUPAC–IUB 1973 recommendations[6] for cyclitols. Thus, racemic I(1,2,5)P_3 consists of Ins(1,2,5)P_3 and Ins(2,3,5)P_3 with the numbering of the NC–IUB 1988 recommendations for *myo*-inositol[7].

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The products of hydrolysis were eluted from a column (40 × 18 cm) of Dowex 1-X8 resin (200–400 mesh) with a linear gradient of 0→1M HCl, as described[17]. In this step, IP_2 was resolved into 2 peaks and IP_3 into 3 peaks.

The components in the IP_2 and IP_3 peaks were fractionated further by elution from a 1-m column of Aminex A-27 (15 μm) with 0.17M HCl.

The collected eluate fractions were analysed for phosphorus by atomic absorption spectroscopy. In order to reduce the number of fractions that had to be analysed, a continuous screening for organic phosphorus was made on a small part of the split eluate, using the sulphosalicylic acid–Fe^{3+} complex[18,19] as the post-column reagent. The reduction in absorbancy of the complex when IP_x competes with the sulphosalicylic acid for Fe^{3+} was detected at 500 nm; 4 vol. of the post-column reagent (0.01% of $FeCl_3 \cdot 6H_2O$ and 0.1% of sulphosalicylic acid) were added to 1 vol. of eluate.

Identification of the IP_x isomers. — In order to avoid isomerisation of IP_x isomers, the acidic eluate was not concentrated but the IP_x was precipitated as the Ca^{2+} salt and then converted into the water-soluble Na^+ salt. The neutral $NaIP_x$ solutions were then concentrated to dryness. Each IP_x was identified by n.m.r. spectroscopy (see ref. 20). I (1,3,5)P_3 was identified by G. W. Mayr (Ruhr-Universität, Bochum, F.R.G.).

RESULTS

Fractionation of the IP_x isomers. — Figure 1 shows the fractionation of IP_2 by chromatography on Dowex 1-X8 resin into $IP_2(1)$ and $IP_2(2)$, and IP_3 into $IP_3(1a)$,

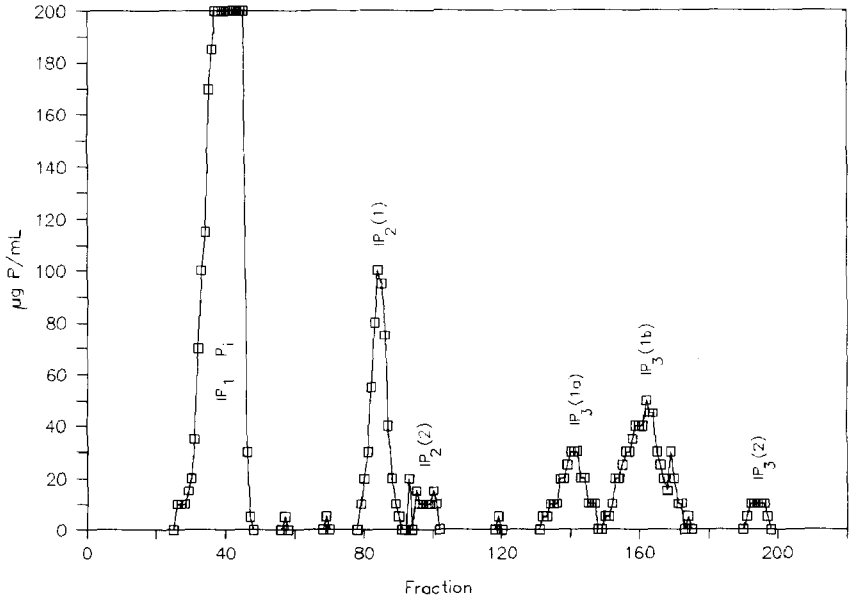

Fig. 1. Hydrolysate (extent of hydrolysis, 30%) of sodium phytate (100 g) eluted from a 10-L column (40 × 18 cm) of Dowex 1-X8 resin (200–400 mesh) with a linear gradient (200 L) of 0→0.2M HCl at 14 L/h (1-L fractions).

IP_3(1b), and IP_3(2). Each of these fractions was precipitated as the Ca^{2+} salt and then converted into the water-soluble Na^+ salt. Only the first half of IP_3 (1b) was treated in this way, since the second half contained only I(1,2,6)P_3 and I(2,4,5)P_3 (Fig. 2).

IP_2(2) and IP_3(2) were pure isomers and were subjected to n.m.r. spectroscopy without further treatment. The remaining IP_x samples were purified further on Aminex A-27 resin. In order to process the amounts needed for n.m.r. spectroscopy in as few runs as possible, the load on the column was favoured at the cost of resolution, and base-line separations were not always obtained. Each sample from the IP_x fractionation step was resolved into 4–6 peaks and the compounds in each peak were isolated as the Na^+ salts as described above.

Identification of the IP_x isomers. — IP_2(2) and IP_3(2) were identified by n.m.r. spectroscopy[20] as I(4,5)P_2 and I(1,5,6)P_3, respectively. The peaks obtained by fractionation of IP_2(1) were shown to contain I(1,3)P_2 + I(4,6)P_2 + I(1,5)P_2 + I(2,5)P_2 + I(1,4)P_2, I(1,2)P_2, and I(1,6)P_2, respectively (Fig. 3). IP_3(1a) was resolved into 6 peaks, 3 of which were well separated. The order of elution was I(1,3,5)P_3 and I(2,4,6)P_3 (well separated), I(1,2,5)P_3, I(1,2,4)P_3, and I(1,3,4)P_3 (close together), and I(1,4,6)P_3 (well separated) (Fig. 4). The first half of IP_3(1b) contained I(1,2,3)P_3, I(1,4,5)P_3, and I(1,2,6)P_3 (Fig. 5).

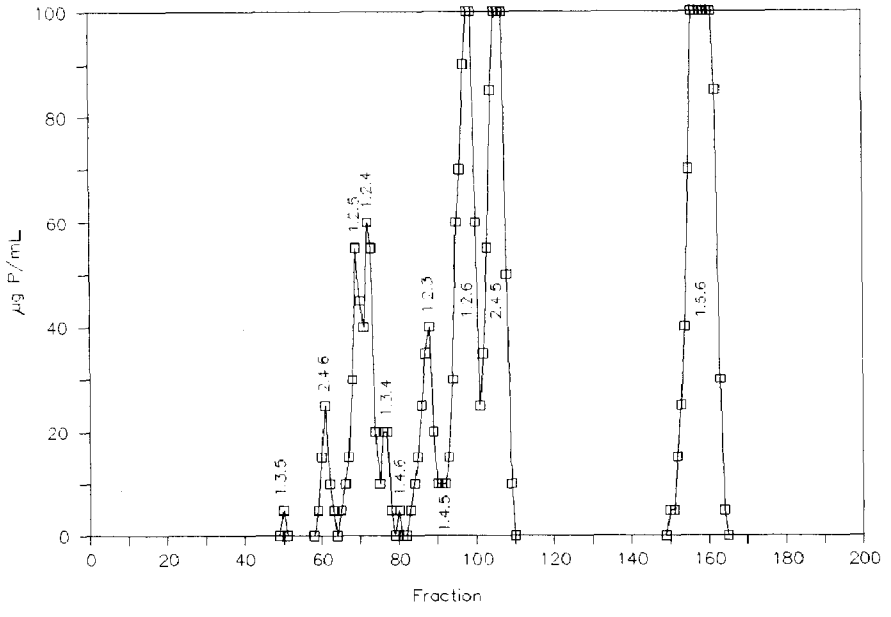

Fig. 2. Fractionation of ~ 60 µmol of IP_3 from hydrolysis of phytate on a column (1 m × 0.7 cm) of Aminex A-27 resin (15 µm) by elution with 0.17M HCl at 20 mL/h (2-mL fractions); ~ 20 µmol of IP_3(2) was added to the original hydrolysate. The numbers associated with each peak (in Figs. 2–5) indicate the locations of the phosphate groups.

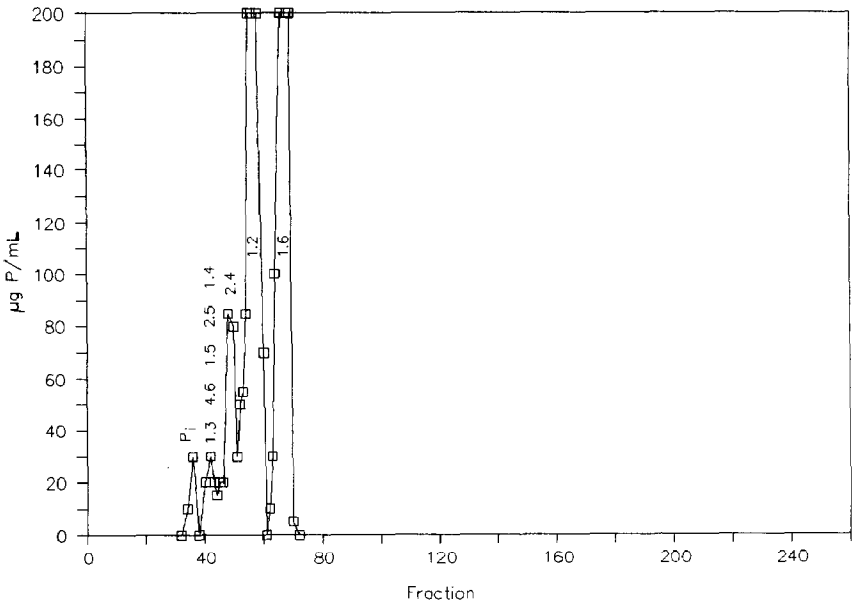

Fig. 3. Fractionation of ~300 µmol of IP_2(1) from Fig. 1 on a column (1 m × 1.5 cm) of Aminex A-27 resin (15 µm) by elution with 0.17M HCl at 100 mL/h (5-mL fractions). The 5 components of the second peak were identified in a larger sample (~100 µmol) of this peak only.

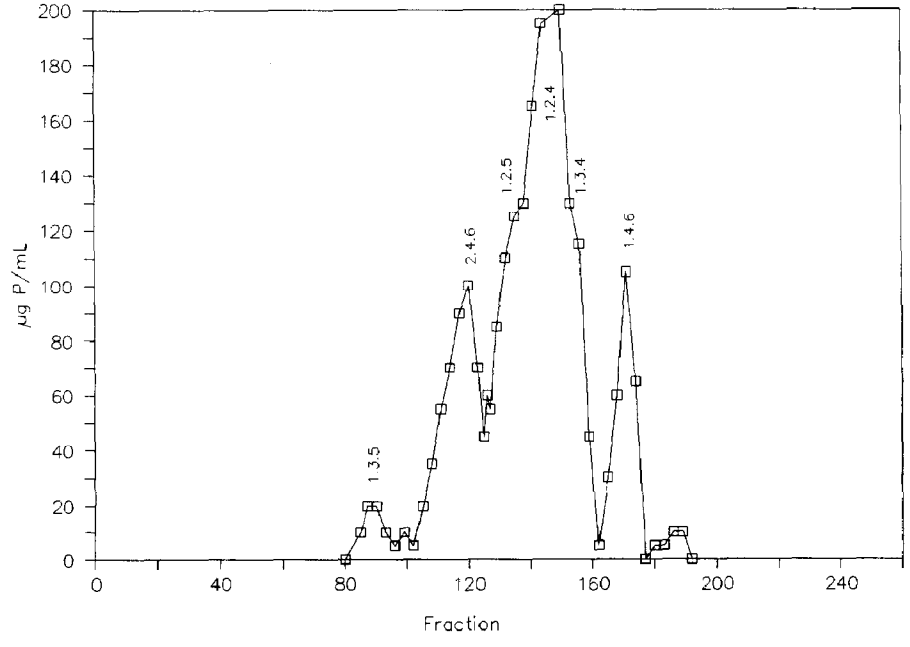

Fig. 4. Fractionation of ~400 µmol of IP_3(1a) from Fig. 1 on Aminex A-27, as in Fig. 3.

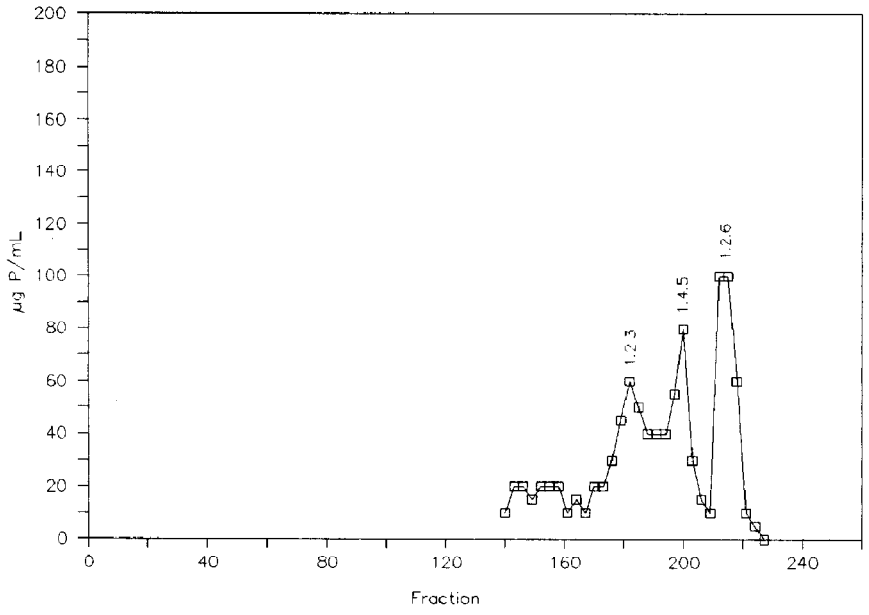

Fig. 5. Fractionation of ~200 µmol of IP_3(1b) (early part) from Fig. 1 on Aminex A-27, as in Fig. 3.

DISCUSSION

All of the (ion-chromatographically distinct) IP_2 and IP_3 isomers except I(4,5,6)P_3 were identified in a chemical hydrolysate of phytic acid. I(4,5,6)P_3, which was not found, was assumed to be present in minor amount and should have been eluted after IP_3(2) from the Dowex 1-X8 resin as reported[11,12]. The concentration of HCl used was optimal for the fractionation of IP_3, but too high for optimal fractionation of IP_2. With a lower concentration of HCl, it should be possible to fractionate the isomers in the first IP_2 peak.

At the low pH of the eluant used, the charge of the IP_x is minimised. Thus, the differences in charge between the various isomers are relatively greater[22] than at higher pH. Also, the isomers are more uniform in charge than at higher pH, where several protonated forms of each isomer exist in equilibrium. Therefore, an acidic eluant such as HCl gives the best resolution of the isomers of IP_3–IP_5 and also eliminates interference by weaker acids, such as carboxylic acids.

Provided that the eluate is concentrated quickly (*in vacuo* or by lyophilisation), no phosphate migration will occur, except with the most susceptible isomers such as I(1,3,5)P_3 and I(2,4,6)P_3, and this can be avoided by precipitating these compounds as the Ca^{2+} salt (see Results).

The elution order of the IP_x isomers from the anion-exchange columns probably reflects the small differences in charge between the individual phosphate groups of the isomers. However, not enough pK_a data are available for comparisons to be made. The

order of elution seems to depend on the number of adjacent phosphate groups and on their positions. Thus, for a given level of phosphorylation, (*a*) the greater the number of adjacent equatorial phosphate groups the stronger will be the affinity for the anion-exchange resin[17], and (*b*) the further away a phosphate group is from the position 2 the greater will be its affinity for the anion-exchange resin. A 2-phosphate, however, is retained more strongly than a 1-phosphate, which reflects the fact that $I(2)P_1$ is more acidic[23] than $I(1)P_1$. Therefore, for example, $I(1,4,5)P_3$ is eluted before $I(2,4,5)P_3$ and $I(1,3,5)P_3$ before $I(2,4,6)P_3$. The order of elution[24,25] $I(1)P_1$, $I(2)P_1$, $I(4)P_1$, and $I(5)P_1$ can be explained in the same way.

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REFERENCES

1 R. H. Michell, *Biochem. Soc. Trans.*, 17 (1989) 1–3.
2 M. Vallejo, T. Jackson, S. Lightman, and M. R. Hanley, *Nature (London)*, 330 (1987) 656–658.
3 D. Carpenter, M. R. Hanley, P. T. Hawkins, T. R. Jackson, L. R. Stephens, and M. Vallejo, *Biochem. Soc. Trans.*, 17 (1989) 3–5.
4 F. Nicoletti, V. Bruno, L. Fiore, S. Cavallaro, and P. L. Canonico, *J. Neurochem.*, 53 (1989) 1026–1030.
5 A. Claxson, C. Morris, D. Blake, M. Sirén, B. Halliwell, T. Gustafsson, B. Löfkvist, and I. Bergelin, *Agents Actions*, 20 (1990) 68–70.
6 *Pure Appl. Chem.*, 37 (1974) 285–297.
7 *Eur. J. Biochem.*, 180 (1989) 485–486.
8 H. Binder, P. C. Weber, and W. Siess, *Anal. Biochem.*, 148 (1985) 220–227.
9 R. F. Irvine, E. E. Änggård, A. J. Letcher, and C. P. Downes, *Biochem. J.*, 229 (1985) 505–511.
10 J. L. Meek and F. Nicoletti, *J. Chromatogr.*, 351 (1986) 303–311.
11 G. W. Mayr, *Biochem. J.*, 254 (1988) 585–591.
12 D. H. Smith and F. E. Clark, *Proc. Soil Sci. Am.*, 16 (1952) 170–172.
13 J. Schormüller and G. Bressau, *Z. Lebensm.-Unters. Forsch.*, 113 (1960) 484–491.
14 C. Grado and C. E. Ballou, *J. Biol. Chem.*, 236 (1961) 54–60.
15 J. A. Shayman and D. M. Be Ment, *Biochem. Biophys. Res. Commun.*, 151 (1988) 114–122.
16 J.-C. Sulpice, P. Gascard, E. Journet, F. Rendu, D. Renard, J. Poggioli, and F. Giraud, *Anal. Biochem.*, 179 (1989) 90–97.
17 D. J. Cosgrove, *Inositol Phosphates*, Elsevier, Amsterdam, 1980, pp. 17–20.
18 H. E. Wade and D. M. Morgan, *Nature (London)*, 171 (1953) 529–530.
19 M. Latta and M. Eskin, *J. Agric. Food Chem.*, 28 (1980) 1315–1317.
20 C. Johansson, J. Kördel, and T. Drakenberg, *Carbohydr. Res.*, 207 (1990) 35–41.
21 B. Q. Philippy and J. M. Bland, *Anal. Biochem.*, 175 (1988) 162–166.
22 D. J. Cosgrove, in W. W. Wells and F. Eisenberg, Jr. (Eds.), *Cyclitols and Phosphoinositides*, Academic Press, New York, 1978, pp. 23–33.
23 H. Bieth, P. Jost, and B. Spiess, *J. Inorg. Biochem.*, 39 (1990) 59–74.
24 I. M. Bird, A. D. Smith, and D. Schulster, *Biochem. J.*, 248 (1987) 203–208.
25 K. A. Wreggett and R. F. Irvine, *Biochem. J.*, 262 (1989) 997–1000.