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Phosphoinositol diphosphates: non-enzymic formation in vitro and occurrence in vivo in the cellular slime mold *Dictyostelium*

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ABSTRACT

When extracts of avian red blood cells were lyophilised, a compound was generated from *myo*-inositol 1,3,4,5,6-pentakisphosphate and ATP which, on the basis of HPLC analysis, limited acid hydrolysis, and ^{31}P and ^{1}H NMR analysis, was identified as tetrakisphospho-*myo*-inositol diphosphate ($InsP_4$-PP). The formation of pyrophosphoryl residues occurs whenever inositol phosphates are lyophilised together with stoichiometric amounts of phosphocreatine and/or nucleoside tri- or di-phosphates. Aqueous extracts of the cellular slime mold *Dictyostelium* contained two major phosphoinositol diphosphates each of which was converted into *myo*-inositol hexakisphosphate by mild acid hydrolysis. The components were shown not to be artifacts and were identified tentatively by HPLC, chemical, and NMR analysis as pentakisphospho-*myo*-inositol diphosphate ($InsP_5$-PP) and tetrakisphospho-*myo*-inositol bis(diphosphate) ($InsP_4$-PP_2).

INTRODUCTION

Since the important role of *myo*-inositol phosphates* in cellular signalling has become known, attention is being focused on the identification of their phosphate metabolites. At present, more than 20 inositol phosphates are known to occur

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* *Abbreviations used*: $InsP_3$, $InsP_4$, $InsP_5$, and $InsP_6$ are *myo*-inositol tris-, tetrakis-, pentakis-, and hexakis-phosphate, respectively (enantiomeric structures are not assigned), and $InsP_3$-PP, $InsP_4$-PP, and $InsP_5$-PP are pyrophosphorylated derivatives; $InsP_7$ denotes pentakisphospho-*myo*-inositol diphosphate(s), $InsP_8$ denotes an $InsP_6$-derived tetrakisphospho-*myo*-inositol bis(diphosphate) ($InsP_4$-PP_2) isolated from *Dictyostelium*, and $InsP_6$* denotes a derivative of $InsP_6$ still containing 6 mol of phosphate per mol of inositol.

naturally in animal cells. Care is necessary in the extraction and analysis of inositol phosphates in order to avoid the generation of artifacts. For example, Ins(1:2cyc,4,5)P_3 is hydrolysed under acidic conditions of extraction[1], to give a mixture of Ins(1,4,5)P_3 and Ins(2,4,5)P_3. Brown et al.[2] observed the formation of inositol methyl phosphates during extraction with methanolic solvents. Acid-catalysed phosphate migration is well known in inositol chemistry and may occur[3] when acidic solutions of inositol phosphates are freeze-dried. Likewise, in phosphoinositides, the non-enzymic formation of phosphorylated derivatives has been reported[4].

We now report that phosphorylation occurs when solutions of inositol phosphates are freeze-dried in the presence of phosphocreatine and nucleotides. Although in vitro experiments demonstrated the possibility of the artificial formation of phosphoinositol diphosphates in extracts from living cells, evidence is presented that indicates these inositol phosphate derivatives to be present in vivo in the cellular slime mold *Dictyostelium*.

EXPERIMENTAL

Nucleoside tri- (ATP, GTP, ITP, UTP, dATP, dCTP, dGTP, and dTTP), di- (ADP and UDP), and mono-phosphates (AMP and UMP) and D-fructose 1,6-bisphosphate were obtained from Boehringer Mannheim, phosphocreatine and Norit A from Serva (Heidelberg), $InsP_6$ (sodium phytate) and 2,3-bisphosphoglycerate from Sigma (München), and [^{3}H]$InsP_6$ from New England Nuclear (Dreieich). The other inositol phosphate standards were prepared and structurally verified as described[3] and $InsP_6$ was further purified by anion-exchange chromatography (see below). All other chemicals and materials were as described[5]. Mono Q columns and Q-Sepharose were obtained from Pharmacia (Freiburg) and analytical grade Dowex resins from BioRad.

Non-enzymic formation of phosphoinositol diphosphates in vitro.—Small-scale reaction mixtures contained 0.25 mM inositol phosphate and 4 mM nucleoside phosphate, phosphocreatine, or pyrophosphate in 400 μL of water. The pH was adjusted to 7.0 with HCl or NaOH. Samples were freeze-dried, resuspended in 100 μL of water, and analysed by HPLC.

For the non-enzymic formation of μmol amounts of phosphorylated $InsP_5$ ($InsP_4$-PP), erythrocytes, isolated by centrifugation (15 min at 900*g*) from 1 L of chicken blood, were extracted with 4 M perchloric acid (250 mL) as described[3], except that 4 mmol of ATP were added to the extract prior to neutralisation with KOH. The precipitated $KClO_4$ was removed, the filtrate was lyophilised, the residue was suspended in water, and the nucleotides were removed by treatment with Norit A.

For the preparation of $\sim$ 50 μmol of phosphorylated $InsP_6$ ($InsP_5$-PP), 1 mmol of $InsP_6$ was dissolved in 0.1 M ATP (150 mL), the pH was adjusted to 7.0, and the solution was freeze-dried.

Culture conditions and harvesting of Dictyostelium *amoebae.—Dictyostelium discoideum*, strain AX2 (ATCC 24397) was grown axenically at 21° as described[6,7]. Cells were harvested by centrifugation for 10 min at 10,000*g*.

Extraction of inositol phosphates from Dictyostelium.—*(a) Under neutral conditions.* A variation of the method of Ishii et al.[8] was used. Packed *Dictyostelium* amoebae (ca. 1 g, $\sim 7 \times 10^8$ cells) were resuspended in 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid]–KOH (3 mL, pH 6.2). The cells were lysed by freezing and mixed with 2 M KCl (0.75 mL), 0.5 M EDTA (0.3 mL, pH 7), and MeOH (7.5 mL). Chloroform (3 mL) was added, and the mixture was shaken vigorously, then left at room temperature for 1 h. Chloroform (4.5 mL), 2 M KCl (1.2 mL), and 0.5 M EDTA (0.3 mL, pH 7) were added, the mixture was centrifuged, and the upper aqueous layer was removed, treated[9] with Norit A in order to remove nucleotides, and then freeze-dried.

(b) Under acidic conditions. For analytical purposes, packed amoebae (1 g, $\sim 7 \times 10^8$ cells) were extracted[9] with perchloric acid. In brief, amoebae were suspended in MES–KOH buffer as in (*a*) and combined with 2 vol. of 2 M perchloric acid. After centrifugation, the supernatant solution was removed, EDTA and acetic acid were added to 5 and 50 mM, respectively, the pH was adjusted to 5 with KOH, the mixture was filtered and treated with charcoal as in (*a*), and the supernatant solution was freeze-dried. In some extracts, freeze-drying was omitted in order to exclude artificial generation of phosphoinositol diphosphates.

For preparative purposes, harvested cells (up to 190 g of packed amoeba) were added to aq 4% (w/v) perchloric acid, mixed vigorously, cooled on ice for 5 min, and centrifuged at 5,000*g* and 4°, and the supernatant solution was neutralised to pH ~ 6.5 with 2 M KOH containing 25 mM EDTA and frozen at −80°. On thawing, the precipitate of $KClO_4$ was removed by centrifugation. In some experiments, [^{3}H]InsP_6 was added to the cells together with the perchloric acid for extraction.

Purification of phosphoinositol diphosphates.—Chromatography on a column (1.5 × 165 cm) of Q-Sepharose was performed as described[3], but strongly acidic conditions were avoided by the use of ammonium acetate instead of HCl as an eluent. Freeze-dried material, containing InsP_4-PP or InsP_5-PP, was diluted with water to a conductivity of 3 mS, applied to the column, and eluted with 3.4 L of a linear gradient 0.4 → 1.3 M ammonium acetate (pH 5.0). Each diphosphorylated inositol phosphate was eluted after InsP_6. The appropriate fractions, detected by total phosphate determination, were combined and freeze-dried to remove ammonium acetate.

InsP_7 extracted from *Dictyostelium* was pre-purified[10] by elution from a column (3 × 10 cm) of Dowex AG 1-X8 (Cl^-) resin (200–400 mesh) with a gradient from 0 to 1.0 M HCl. InsP_7 was eluted immediately after the large InsP_6 peak. The appropriate fractions were combined and treated with $Ba(OH)_2$, and the precipitated barium salt of InsP_7 was collected, converted into the free acid by treatment with an excess of Dowex AG 50 (H^+) resin, and neutralised with NaOH. The

material was purified further by chromatography on Q-Sepharose as described above. When [^{3}H]InsP_6 had been added to the extract, an aliquot of each fraction was mixed with liquid scintillator and counted for radioactivity.

Analysis of inositol phosphates by HPLC–MDD.—Samples were analysed by HPLC with metal dye detection (MDD) as described[3,5,9]. Only the solid-phase extraction, performed with part of the *Dictyostelium* extracts, was modified in order to avoid acidic conditions. After treatment[9] with charcoal, each sample was diluted with water to 50 mL and added to a disposable column of Q-Sepharose (1 mL, AcO^- form). After two washings with 3 mM HCl (3 mL), the inositol phosphates were eluted with 1.8 M ammonium acetate (5 mL). This eluate was freeze-dried until no carrier salt remained. A solution of the residue in the application buffer (2.2 mL) containing 2.5 mM NaOAc and mM NaF (pH 6.0) was stored at −30°. The separation systems used consisted of a guard column (0.5 × 5 cm) and a main column (0.5 × 20 cm) packed with Mono Q, or a column (0.5 × 10 cm) packed with Mono Q. The long-column system was developed with the HCl gradient described[9]. For analysis of *Dictyostelium* extracts, this gradient was extended by prolonging the time at 100% B to 17 min. The shorter column was developed with two different HCl gradients prepared from A, 200 μM HCl–14 μM YCl_3; and B, 0.5 M HCl–14 μM YCl_3. The separation of $InsP_5$-PP isomers involved gradient I at 1.5 mL/min: 0% → 30% B in 0.1 min, 30% → 70% B in 8 min, 70% → 75% B in 7 min, 75% → 100% B in 12 min, end. The post-column dye reagent was pumped at 0.75 mL/min. The separation of the $InsP_3$-PP isomers involved gradient II at 1.5 mL/min: 0% → 10% B in 0.1 min, 10% → 35% B in 20 min, 35% B in 20 min, 35% → 50% B in 5 min, 50% → 65% B in 3 min, 65% → 100% B in 2 min, 100% B for 7 min, end. Alternatively, the small column was developed with a KCl gradient at pH 8.0 and 1.5 mL/min using *A*, 10 mM triethanolamine and *B*, 10 mM triethanolamine–0.4 M KCl. The post-column dye reagent contained 2 mM ammonium acetate (pH 5.0) with 300 μM 4-(2-pyridylazo)resorcinol and 45 μM YCl_3. The linear gradient was formed as follows: 0% → 25% B in 0.1 min, 25% → 100% B in 35 min, end. The dye reagent was delivered at 0.75 mL/min.

Hydrolysis of InsP4-PP.—*(a) With acid.* $InsP_4$-PP (0.5 μmol), isolated as described above, was applied to a column (1.5 × 2 cm) of Q-Sepharose (Cl^--form) and rapidly eluted with 0.5 M HCl. Fractions (0.5 mL) were assayed for total phosphate. $InsP_4$-PP was eluted after small amounts of hydrolysis products. The combined fractions containing $InsP_4$-PP were diluted to 0.1 M HCl, and (*a*) analysed for $InsP_5$ by HPLC and assayed for total and inorganic phosphate, and (*b*) boiled under reflux for 15 min, neutralised, and analysed as in (*a*) after hydrolysis.

(b) With alkali. Aliquots (~ 10 nmol) of $InsP_4$-PP in 0.1 M NaOH were boiled under reflux for up to 15 min, then neutralised with HCl, and analysed as described above.

Time course of the acid hydrolysis of InsP5-PP.—Each sample contained 30 nmol

of $InsP_5$-PP in 0.1 M HCl–10 mM acetic acid (150 μL). Hydrolysis was carried out at 80° for 0–100 min and was terminated by the addition of 2 M triethanolamine (10 μL). Samples were assayed for total and inorganic phosphate. $InsP_6$ formed was quantified by HPLC employing the acidic system (see above). Data points were fitted to a curve of first-order kinetics by applying the equation $y = A(1 - e^{-Bx})$.

Acid hydrolysis of Dictyostelium *extracts.*—Charcoal-treated and solid-phase extracted samples in the application buffer[9] were mixed with an equal volume of 2 M trichloroacetic acid, kept at 100° for 7 and 15 min, then cooled in ice, extracted with ether (4 ×), and analysed by HPLC.

Formation and purification of $InsP_6$-II.*—A solution of $InsP_5$-PP (~ 300 nmol), prepared and purified as described above, in 0.1 M HCl (700 μL) was boiled under reflux for 15 min, neutralised, and applied to the short Mono Q column, which was developed with the acidic gradient I (see above) except that the eluent did not contain YCl_3 and no post-column reagent was added to the eluate. When the elution of $InsP_6$ was complete, the eluate was collected for 2 min (14.5 → 16.5 min after the start of the gradient). The fraction which contained 22 nmol of $InsP_6$*-II was diluted with water and freeze-dried to remove HCl.

Treatment of $InsP_6$-II with alkali.*—Aliquots containing 6 nmol of $InsP_6$*-II were autoclaved for 45 min in M NaOH (200 μL) in sealed tubes at 120°, then neutralised, and assayed for inorganic phosphate. Portions corresponding to 2 nmol of $InsP_6$* were analysed by HPLC–MDD.

Analysis for total and inorganic phosphate.—A scaled-down modification of the procedure described[11,12] was used.

NMR measurements.—Inositol phosphates were prepared for NMR spectroscopy as described[3,13]. For ^1H NMR spectra, the sample volume was 700 μL, and the standardisation was done as described[14]. For ^{31}P NMR spectra, standardised against external aq 85% orthophosphoric acid, the sample volume was 3.5 mL (in 10-mm diameter tubes), and the spectra were obtained without proton decoupling. Samples were adjusted[14] to a pH* (D_2O) of 6.0 or 9.0 and were measured at 300 K (^{31}P) or 333 K (^1H).

RESULTS AND DISCUSSION

Non-enzymic phosphorylation of inositol phosphates.—Non-enzymic phosphorylation of inositol phosphates was noticed first during experiments with avian erythrocytes stimulated by ATP. HPLC of the inositol phosphates subsequently extracted revealed an unknown compound (*X*) which was eluted after $InsP_6$ (data not shown). This product was an artifact since it was formed only when extracts containing large proportions of $InsP_5$ and ATP were freeze-dried. In fact, $InsP_5$ and ATP, lyophilised under conditions as specified in the Experimental, were found to be the only components necessary for the formation of the highly polar compound. A larger amount of *X* was prepared by freeze-drying an extract of avian erythrocytes supplemented with ATP and isolated by anion-exchange chro-

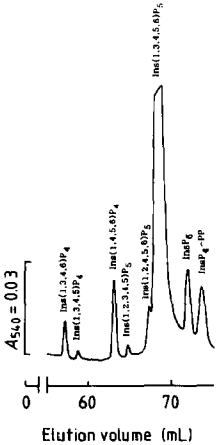

Fig. 1. Inositol phosphates in ATP-supplemented, freeze-dried avian erythrocyte extracts. An extract from chicken blood (1 L) was mixed with 4 mmol of ATP and freeze-dried. The residue was dissolved in water and an aliquot corresponding to 50 μL of erythrocytes was analysed by HPLC–MDD on a Mono Q column (0.5×25 cm) with an acidic eluent (see Experimental). Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5 are products of phosphate migration derived from the large amount of Ins(1,3,4,5,6)P_5 (~ 150 nmol) during chromatography.

matography. Fig. 1 shows an HPLC analysis of the freeze-dried extract. The elution profile was essentially identical to those of extracts of ATP-stimulated erythrocytes.

Acid hydrolysis (0.1 M HCl, 100°, 15 min) of *X* effected complete conversion into $InsP_5$ and P_i, but no hydrolysis occurred under alkaline conditions. HPLC revealed Ins(1,3,4,5,6)P_5 to be the major $InsP_5$ product. The presence of two other isomers of $InsP_5$ that co-chromatographed with Ins(1,2,4,5,6)P_5 and Ins(1,2,3,4,5)P_5 was consistent with acid-catalysed phosphate migration in Ins(1,3,4,5,6)P_5 and was confirmed by treatment of authentic Ins(1,3,4,5,6)P_5 with acid. The molar ratio of $InsP_5$ and P_i generated was shown by HPLC and inorganic phosphate analysis to be 1 : 1.

On the basis of these results, *X* was identified tentatively as tetrakisphosphoinositol diphosphate ($InsP_4$-PP, *myo*-inositol tetrakisphosphate diphosphate).

The conversion of $InsP_5$ into $InsP_4$-PP varied markedly (2–15%) between samples, apparently reflecting the efficiency of the freeze-drying device. ATP could be substituted by other nucleoside triphosphates and also by phosphocreatine and phosphoenolic pyruvate, the latter two phosphate donors giving up to 30% conversion. Smaller yields of phosphorylation products were observed when nucleoside diphosphates or pyrophosphate were used as the phosphate donors and none occurred with nucleoside monophosphates or sugar phosphates. Divalent metal ions, essential[4] for non-enzymic phosphorylation of phosphoinositides, impaired the yield of phosphorylation products. Ins(1,4,5)P_3, Ins(1,5,6)P_3, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4, Ins(1,3,4,5)P_4, and $InsP_6$, and even Fru(1,6)P_2, underwent non-enzymic phosphorylation in a manner similar to that of Ins(1,3,4,5,6)P_5,

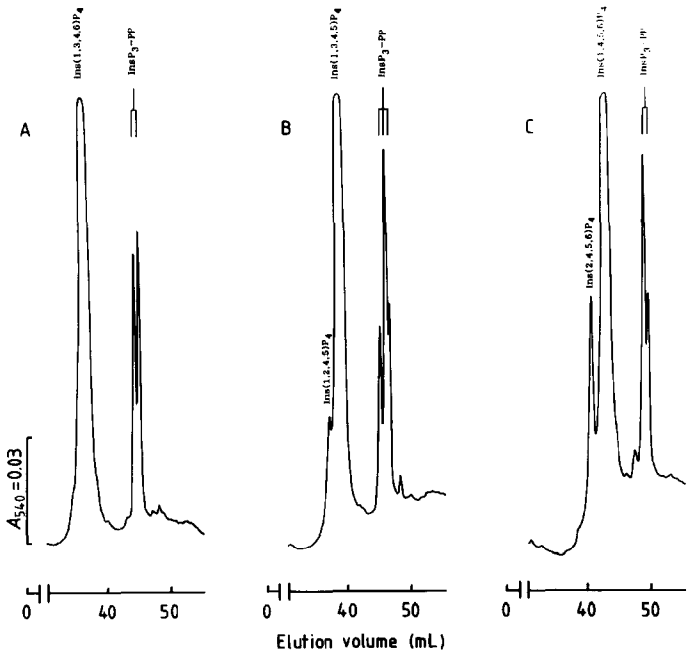

Fig. 2. HPLC of products of non-enzymic phosphorylation of A, Ins(1,3,4,5)P4; B, Ins(1,3,4,6)P4; and C, Ins(1,4,5,6)P4 on a Mono Q column (0.5×10 cm) eluted with acidic gradient II (see Experimental): Ins(2,4,5,6)P_4 and Ins(1,2,4,5)P_4 are products of phosphate migration in Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4, respectively, not exceeding 4% of the main isomer of $InsP_4$ (note the saturation of the detection system).

but gave complex mixtures of phosphorylated compounds that were eluted in HPLC after the corresponding parent compound. Fig. 2 shows the patterns of the products of non-enzymic phosphorylation of several isomers of $InsP_4$.

The kinetics of hydrolysis of $InsP_5$-PP, the product of non-enzymic phosphorylation of $InsP_6$, in 0.1 M HCl at 80° was studied in more detail. The rate of the generation of P_i and $InsP_6$ during 100 min (data not shown) accorded precisely with a first-order curve with a half-life of 28 min. This result indicated a one-step reaction in which a pyrophosphate bond was hydrolysed without the formation of intermediates.

Natural occurrence of phosphoinositol diphosphates in Dictyostelium.—Europe-Finner et al.[15,16] reported a highly polar inositol phosphate derivative in *Dictyostelium* extracts, which was eluted after $InsP_6$ in anion-exchange HPLC, and speculated that it might be a phosphoinositol diphosphate formed in vivo. In order to confirm this assumption, inositol phosphates were extracted from *Dictyostelium* and analysed by HPLC–MDD (Fig. 3). Two inositol phosphate derivatives were eluted after $InsP_6$ from the Mono Q column, and were tentatively termed $InsP_7$ and $InsP_8$. The concentrations of these compounds (in nmol/g of wet-packed cells) were 302–444 for $InsP_6$, 26–45 for $InsP_7$, and 69–114 for $InsP_8$. On the basis of a

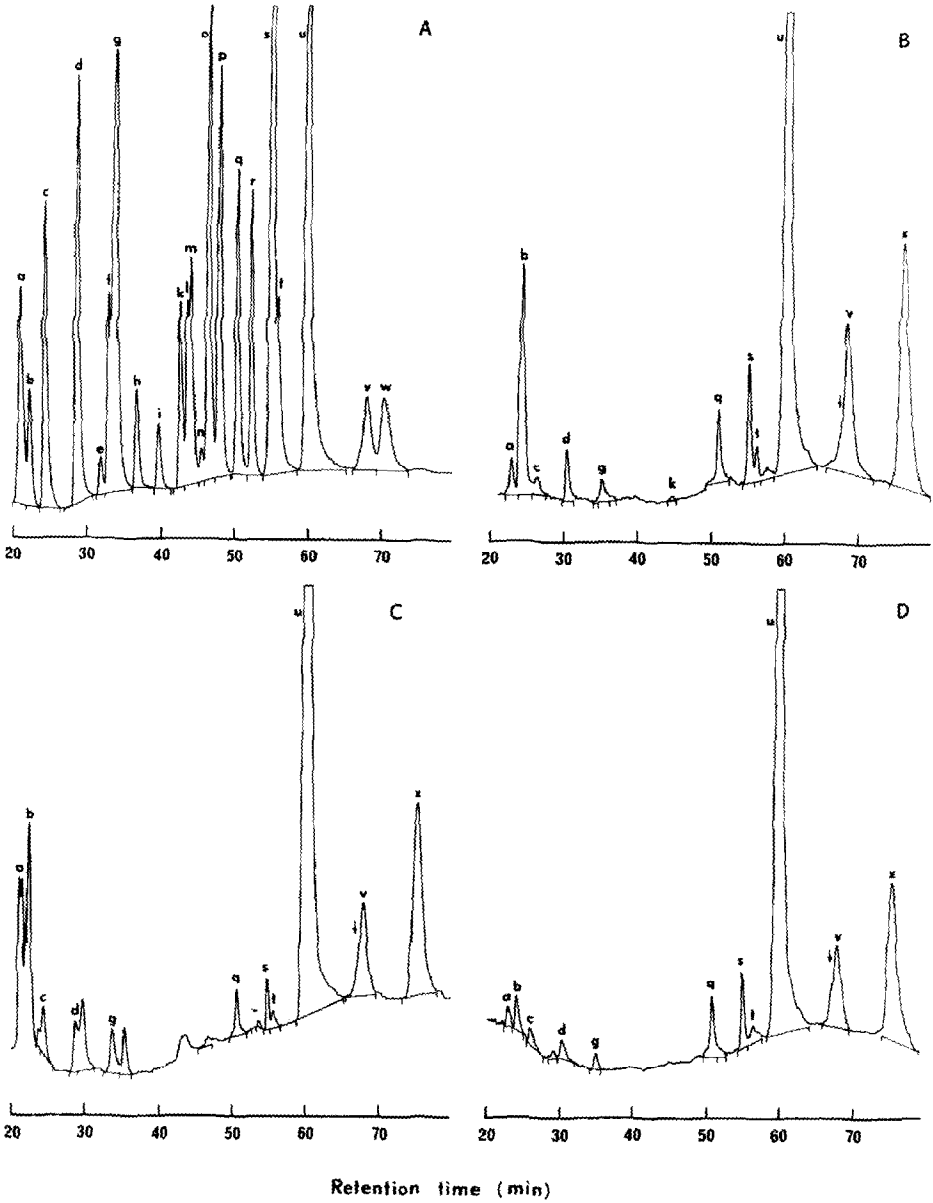

Fig. 3. HPLC of inositol phosphates extracted from *Dictyostelium* on a Mono Q column (0.5×25 cm), using an acidic eluent (see Experimental): A, standard mixture containing a, $InsP_2$; b, Fru(1,6)P_2; c, PPi; d, 2,3-bisphosphoglycerate; e, Ins(1,3,5)P_3 and Ins(2,4,6)P_3; f, Ins(1,2,4)P_3, Ins(1,3,4)P_3, Ins(1,2,5)P_3, and Ins(1,4,6)P_3; g, Ins(1,2,3)P_3, Ins(1,2,6)P_3, Ins(1,4,5)P_3, and Ins(2,4,5)P_3; h, Ins(1,5,6)P_3; i, Ins(4,5,6)P_3; k, Ins(1,2,3,5)P_4 and Ins(1,2,4,6)P_4; l, Ins(1,2,3,4)P_4 and Ins(1,3,4,6)P_4; m, Ins(1,2,4,5)P_4; n, Ins(1,3,4,5)P_4; o, Ins(1,2,5,6)P_4; p, Ins(2,4,5,6)P_4; q, Ins(1,4,5,6)P_4 and Ins(1,2,3,4,6)P_5; r, Ins(1,2,3,4,5)P_5; s, Ins(1,2,4,5,6)P_5; t, Ins(1,3,4,5,6)P_5; u, $InsP_6$; v, $InsP_5$-PP-I; w, $InsP_5$-PP-II; B, non-lyophilised, charcoal-treated perchloric acid extract from 48 mg wet-packed cells: x, $InsP_8$; ↓, $InsP_5$-PP-III; C, charcoal-treated and solid-phase extracted chloroform–methanol–water extract from 70 mg of wet-packed cells; D, charcoal-treated and solid-phase extracted perchloric acid extract from 47 mg of wet-packed cells. For standardisation, a ratio for $InsP_5$-PP-I (plus $InsP_5$-PP-III) to $InsP_5$-PP-II of 1:1 was employed. For standardisation of $InsP_8$, a detection sensitivity of 8/7 that of $InsP_5$-PP was assumed. Full-scale absorbance corresponds to $A_{546nm} = 0.1$.

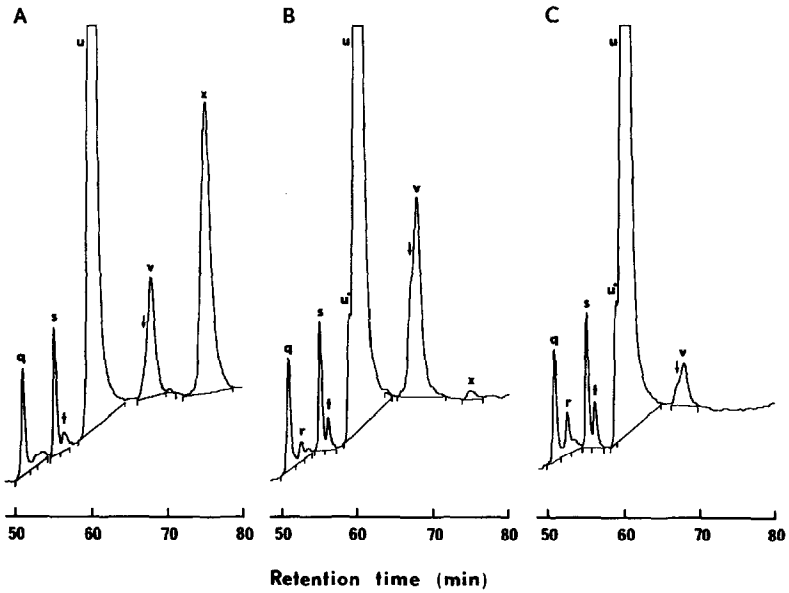

Fig. 4. Limited acid hydrolysis of inositol phosphates extracted from *Dictyostelium*. The inositol phosphates were perchloric acid-extracted, charcoal-treated, and solid-phase extracted (see Experimental) from 47 mg of wet-packed cells without (A), and after hydrolysis (M trichloroacetic acid, 100°) for 7 min (B) and 15 min (C). HPLC conditions as in Fig. 3; u*, $InsP_6$*-I (see Fig. 6). Only the final part of each chromatogram is depicted.

mean cell volume of 0.6 pL, the corresponding intracellular concentrations were ≤ 0.7, ≤ 0.1, and ≤ 0.2 mM, respectively. The presence of these compounds was independent of whether the extracts were obtained by acidic (Figs. 3B and 3D) or neutral organic–aqueous extraction (Fig. 3C), or were freeze-dried during work up (Figs. 3C and 3D) or not (Fig. 3B). Furthermore, when cells were extracted in the presence of added [^{3}H]$InsP_6$, practically no ^{3}H could be detected in these compounds. These data suggest that $InsP_7$ and $InsP_8$ were present in vivo and were not artifacts.

Some of the extracts were submitted to conditions known to destroy the energy-rich phosphates. Thus, treatment with boiling M trichloroacetic acid for 7 min hydrolysed most of the $InsP_8$ (Fig. 4B), and the peaks for $InsP_7$ and $InsP_6$ increased. After 15 min (Fig. 4C), most of $InsP_7$ had disappeared and the peak for $InsP_6$ had increased further. A leading shoulder (marked u*) in the peak of $InsP_6$ appeared during the course of hydrolysis. The leading shoulder in the $InsP_7$ peak indicated the existence of two isomers of $InsP_7$ in the original extract. During the hydrolysis, apparently all $InsP_8$ was degraded via these two species of $InsP_7$ (in about equal proportion) to acid-stable $InsP_6$ and an unknown minor component ($InsP_6$*-I, see below) which was eluted close to $InsP_6$.

Non-enzymic phosphorylation of $InsP_6$ yielded two chromatographically separable components of $InsP_5$-PP ($InsP_5$-PP-I and $InsP_5$-PP-II) in the molar ratio of

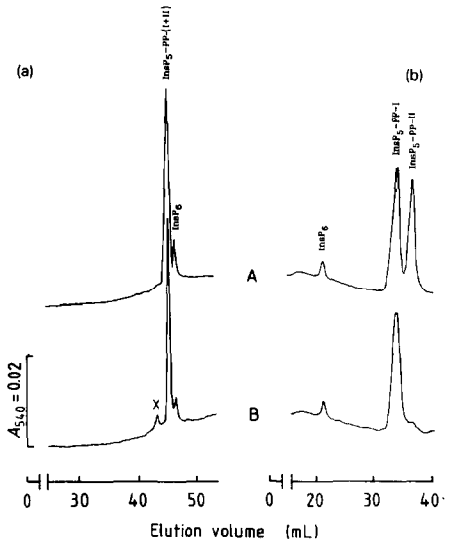

Fig. 5. HPLC of $InsP_5$-PP and $InsP_7$ (prepared and isolated as described in the Experimental). Aliquots (1–4 nmol) of $InsP_5$-PP (A) and $InsP_7$ (B) were analysed on a Mono Q column (0.5 × 10 cm) employing a slightly alkaline KCl gradient (a) or, alternatively, a HCl gradient (b) (gradient I, see Experimental). X indicates an unidentified contaminant.

~ 1:1, but practically no $InsP_8$-like compound (Fig. 5). This markedly differing spectrum of products also indicated that the $InsP_7$ and $InsP_8$ isolated from *Dictyostelium* are not artifacts. On elution under acidic conditions, $InsP_5$-PP-I and $InsP_5$-PP-II were well separated after $InsP_6$ (Fig. 5b, chromatogram A). The peak designated $InsP_5$-PP-I had a leading shoulder which indicated the presence of a minor third isomer ($InsP_5$-PP-III). When employing a slightly alkaline KCl gradient, the compounds migrated closely together before $InsP_6$ (Fig. 5a, chromatogram A). $InsP_5$-PP-I was eluted now after $InsP_5$-PP-II, but this exchange of positions was visible only when small amounts of $InsP_5$-PP were analysed (cf. Fig. 6). When chromatograms of analytical extracts from *Dictyostelium* were compared with standard chromatograms containing these synthetic $InsP_5$-PP species, $InsP_7$ co-eluted with $InsP_5$-PP-I (cf. Fig. 3) and contained a variable proportion of $InsP_5$-PP-III.

As Fig. 5a demonstrates, the main component of $InsP_7$ isolated from *Dictyostelium* co-chromatographed with $InsP_5$-PP-I, and a minor proportion of $InsP_5$-PP-II was present. Differing extraction and work-up procedures for the large-scale isolation of $InsP_7$ (presumably having led to artificial formation of some $InsP_5$-PP-II) may be responsible for this difference from the $InsP_7$ found in analytical extracts.

$InsP_7$ and $InsP_5$-PP had identical acid labilities which were similar to that of $InsP_4$-PP. After boiling for 15 min in 0.1 M HCl, $InsP_7$ or $InsP_5$-PP was converted almost completely into P_i, $InsP_6$ and to $< 5\%$ of two acid- and alkali-labile by-products termed $InsP_6$*-I and $InsP_6$*-II. On elution under acidic conditions from

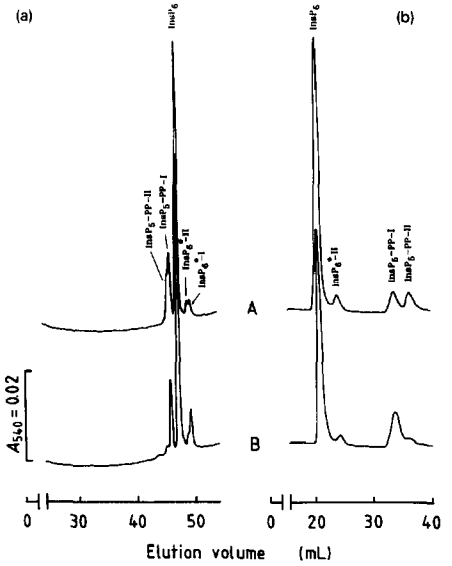

Fig. 6. HPLC of partially hydrolysed $InsP_5$-PP and $InsP_7$. Aliquots (1–4 nmol) of the compounds analysed in Fig. 5 in 0.1 M HCl were boiled under reflux for 5 min. The hydrolysates of $InsP_5$-PP (A) and $InsP_7$ (B) were analysed by HPLC employing a KCl gradient (a) or a HCl gradient (b) (see Fig. 5). $InsP_6$*-I and $InsP_6$*-II represent two isomeric forms of $InsP_6$* (see Results and Discussion).

the 10-cm Mono Q column, only $InsP_6$*-II was eluted after $InsP_6$ (Fig. 6), whereas $InsP_6$*-I was co-eluted with $InsP_6$. However, on the 25-cm column, $InsP_6$*-I was eluted slightly ahead of $InsP_6$ (data not shown). In the acid-treated *Dictyostelium* extracts, only $InsP_6$*-I appeared to have been formed (see Fig. 4). Likewise, when isolated $InsP_7$ (mainly corresponding to $InsP_5$-PP-I) was hydrolysed, this earlier-eluting species (co-eluted with $InsP_6$ in Fig. 6b) appeared to be preponderant. With the hydrolysate of $InsP_5$-PP, elution under alkaline conditions from the 10-cm Mono Q column (Fig. 6a) indicated that $InsP_6$*-I and $InsP_6$*-II (having a changed order of elution) were formed in a molar ratio ($\sim$ 1 : 1) similar to that of $InsP_5$-PP-I and $InsP_5$-PP-II. Thus, it is concluded that $InsP_6$*-I is derived mainly from $InsP_5$-PP-I and $InsP_6$*-II mainly from $InsP_5$-PP-II. This finding adds further proof to the proposal that the $InsP_7$ consists of isomers of $InsP_5$-PP, which are also formed non-enzymically in vitro. When $InsP_6$*-II was purified and autoclaved in M alkali (45 min, 120°), conditions where $InsP_6$ was not hydrolysed, it was converted exclusively into $InsP_6$ without releasing any P_i. The structures of $InsP_6$*-I and $InsP_6$*-II remain to be determined but their behaviour on hydrolysis suggests that they may be derivatives of $InsP_6$ that contain cyclic pyrophosphate groups.

Assignment of the structure of the phosphoinositol diphosphates.—Unequivocal proof of the presence of pyrophosphoryl residues in $InsP_7$ and $InsP_8$ came from ^{31}P NMR spectroscopy. Fig. 7 shows the spectra of $InsP_5$-PP recorded at pH* 6.0 and 9.0. Each spectrum shows typical[17] upfield-shifted resonances of α- and β-phosphates of a pyrophosphoryl residue and an unresolved group of phosphomo-

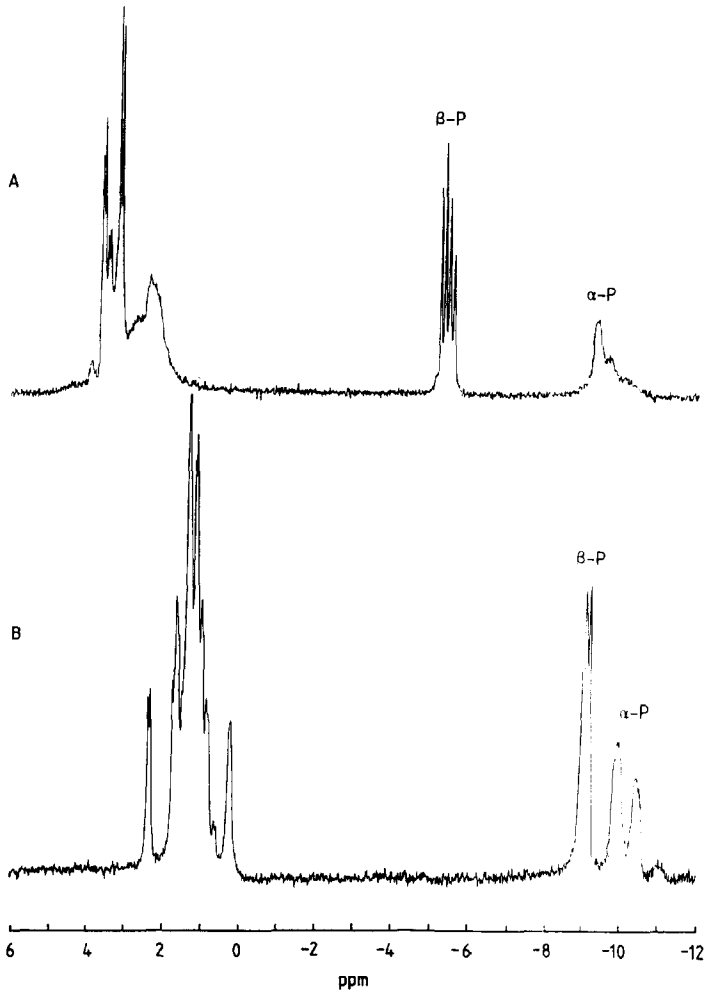

Fig. 7. ^{31}P NMR spectra of a solution of $InsP_5$-PP (33 μmol) in D_2O recorded at a pH* of 9.0 (A) and 6.0 (B). α-P and β-P denote the resonances of α- and β-phosphates of pyrophosphoryl residues. Phosphomonoester resonances were not assigned.

noester resonances at 0.2–3.6 ppm. Although not resolved completely in each spectrum, two major and a minor resonance were observed for each α- and β-P which indicated the presence of three different compounds. A $^2J_{POP}$ value for the P_α–O–P_β coupling of 17.7–19.6 Hz accords with published data of diphospho compounds[17]. A further, non-resolved splitting of the α-P resonance (see Fig. 8) is due to coupling with a vicinal ring proton with a $^3J_{POCH}$ value of $\sim$ 10 Hz. As expected from the behaviour of the two adjacent phosphates on protonation[17], only the resonances of the β-P showed a marked upfield shift (-5.4 to -9.1 ppm) upon reduction of pH* from 9.0 to 6.0, whereas those of α-P were shifted only

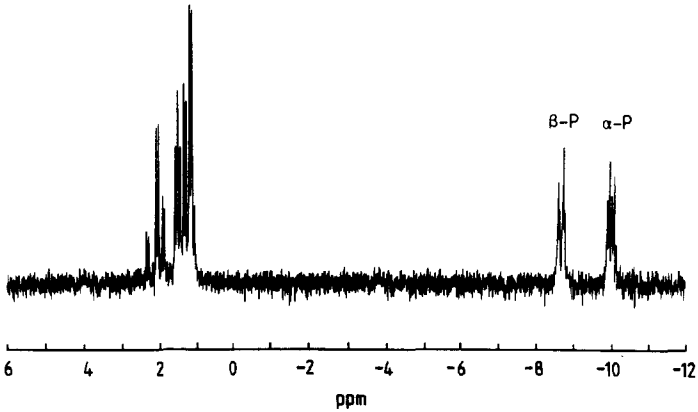

Fig. 8. ^{31}P NMR spectrum of a solution of $InsP_4$-PP (1 μmol) in D_2O measured at pH* 6.0. α-P and β-P indicate the resonances of the α- and β-phosphate group, respectively, of the pyrophosphoryl residue. Other resonances were not assigned.

slightly (−9.5 to −10.2 ppm); the phosphomonoester resonances where shifted upfield by ~1.5 ppm.

$InsP_4$-PP gave a ^{31}P NMR spectrum with corresponding α- and β-P resonances (Fig. 8), with one major and a minor proportion of a second species as deduced from the two β-P resonances. The high resolution of this spectrum allowed a $^3J_{POCH}$ value of the α-P resonances to be determined as 10.0 Hz in addition to a $^2J_{POP}$ value of 20.3 Hz. Among the phosphomonoester resonances, six major signals (d) could be discriminated at 2.05, 1.52, 1.45, 1.29, 1.14 and 1.13 ppm. Since about a third of the material was the symmetrical parent compound Ins(1,3,4,5,6)P_5 (which gave rise to 3 d with intensities in the ratios ~2:2:1), two of those major resonances might be due to that contaminant. The remaining four resonances indicated that the major $InsP_4$-PP was asymmetric and could not be Ins(1,3,4,6)P_4-(5)PP. All phosphomonoester resonances exhibited a $^3J_{POCH}$ value of 9.6 ± 0.3 Hz.

The position of non-enzymic phosphorylation of the inositol phosphates could be elucidated by HPLC (see above), and further clarified for synthetic $InsP_5$-PP by its ^1H NMR spectrum (Fig. 9). Most resonances were crowded between 4.1 and 4.6 ppm and were not assigned. However, there were three isolated resonances between 4.7 and 4.9 ppm, characteristic for H-2 coupled with the phosphorus of an adjacent phosphate residue[14]. The chemical shift of the H-2 resonance marked 2A is identical to that of H-2 of $InsP_6$, which was also present in a small proportion in the $InsP_5$-PP preparation (see Fig. 3). Resonances 2B and 2C apparently correspond to H-2 of the major isomers of $InsP_5$-PP. Due to the proximity of the pyrophosphoryl moiety, H-2 of $InsP_5$-PP is likely to be more deshielded than H-2 of $InsP_6$. However, such deshielding will occur only when the pyrophosphoryl residue is attached to C-2 or C-1/C-3. Since two strongly downfield-shifted H-2 resonances are present, both of these phosphorylation positions are likely. The

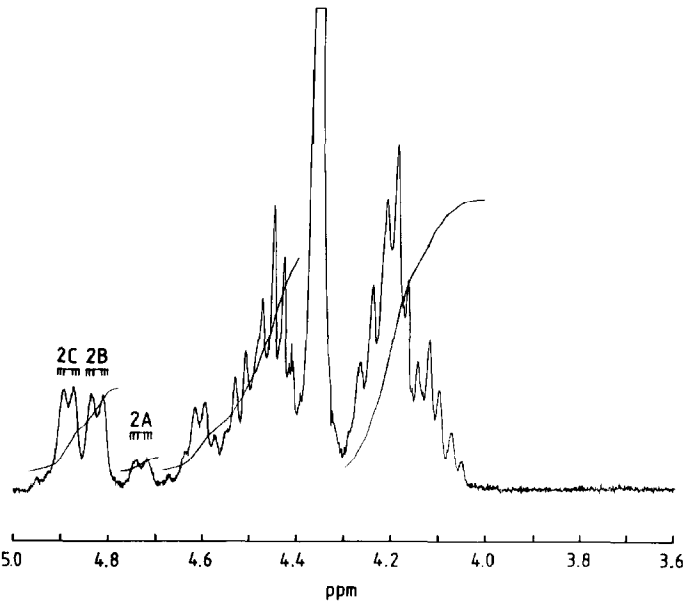

Fig. 9. ^{1}H NMR spectrum of a solution of $InsP_5$-PP (12 μmol) in D_2O recorded at a pH* of 6.0; 2A, H-2 of $InsP_6$; 2B and 2C, H-2 of the two isomers of $InsP_5$-PP. The large resonance at 4.38 ppm is due to HDO.

most downfield-shifted resonance (2C) is assigned tentatively to an $InsP_5$-PP isomer pyrophosphorylated at C-2 and the less downfield-shifted resonance 2B to an isomer pyrophosphorylated at C-1 or C-3. It is concluded that C-2 of $InsP_6$ is the favoured site for the non-enzymic formation of a pyrophosphoryl group. When C-2 is not phosphorylated, as in Ins(1,3,4,5,6)P_5, only one major product is detectable by HPLC and NMR analysis, probably consisting of a racemic $InsP_4$-PP with a pyrophosphoryl residue at C-1 or C-3. Due to steric reasons, phosphate groups at the other positions of $InsP_5$ and $InsP_6$ seem to be less favourable for accepting a diphosphoryl moiety. Phosphoryl residues other than those at C-2 appear to allow an easy accommodation of the bulky pyrophosphoryl group only if there is at least one neighbouring hydroxyl group. This view is supported by the number of main products observed on non-enzymic phosphorylation of isomers of $InsP_4$ (cf. Fig. 2). Phosphorylation of Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4 gave two and three separable isomers of $InsP_3$-PP, respectively. In Ins(1,3,4,6)P_4, all four phosphoryl residues should act as potential targets for a non-enzymic phosphorylation. HPLC revealed two phosphorylation products in the molar ratio $\sim 1:1$ which is consistent with the formation of the expected two pairs of enantiomers.

The ^{31}P NMR spectrum of purified $InsP_7$ (Fig. 10) demonstrated a pattern similar to that observed for $InsP_5$-PP at a similar pH* (Fig. 7A). Mainly the β-P resonances revealed the existence of two isomers of $InsP_5$-PP with one preponderant species. The chemical shifts were in agreement with those of synthetic $InsP_5$-PP. Purified $InsP_8$ (Fig. 10B) showed no additional phosphate resonance at

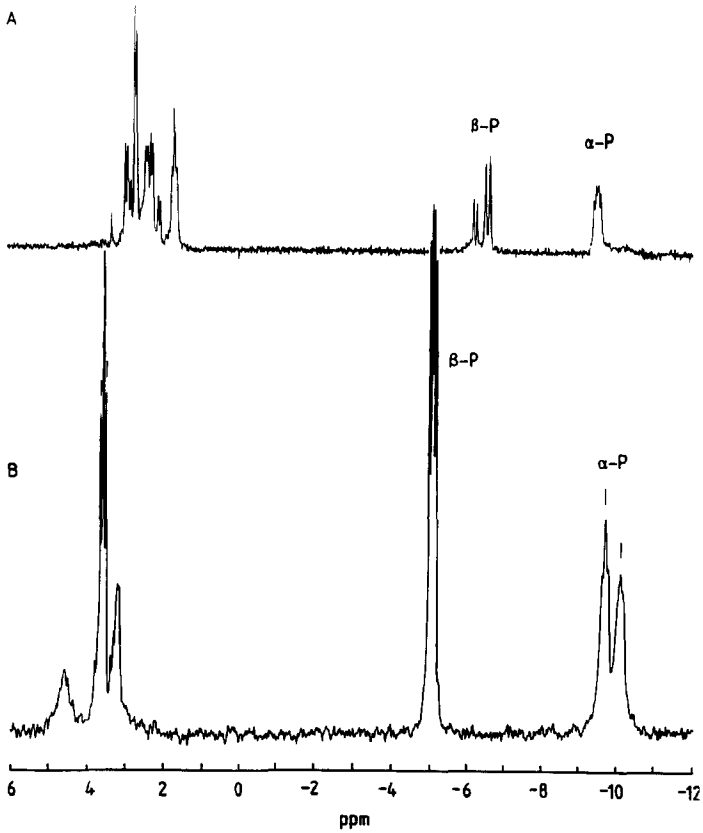

Fig. 10. ^{31}P NMR spectra of solutions in D_2O of $InsP_7$ (4.7 μmol) at pH* 8 (A) and $InsP_8$ (8 μmol) at pH* 9. (B). α-P and β-P indicate the resonances of the α- and β-phosphorus nuclei, respectively, of the pyrophosphoryl residues. Phosphomonoester resonances were not assigned.

~ -20 ppm as would be expected for a 'middle phosphate' in an ATP-like triphosphate[17]. Instead, two α-P resonances at -9.7 and -10.1 ppm and two β-P resonances at -5.0 and -5.1 ppm of similar intensities were detectable. Assignments to α-P and β-P of diphosphoryl residues were confirmed by the above-discussed pH dependencies of chemical shifts and observed coupling patterns. Thus, the structure of $InsP_8$ could be assigned to one single asymmetric isomer or, less likely due to the identical intensities of the pairs of α-P and β-P resonances, to two different symmetrical isomers of tetrakisphospho-*myo*-inositol bis(diphosphate).

Thus, novel inositol phosphates, phosphoinositol diphosphates, can be generated artificially in extracts from living cells when energy-rich phosphates are present in high concentrations and the extracts are lyophilised. This phenomenon must be taken into account in the analysis of inositol phosphates. In order to circumvent this problem, it is suggested that cell extracts which contain high

concentrations of nucleotides should be treated with charcoal when lyophilisation is necessary. Phosphocreatine, if present in high concentration, can be destroyed by incubation of acid extracts for 20–40 min at 30°, a condition not affecting inositol phosphates. Only when acid-labile sugar bisphosphates are also to be determined is this treatment impossible.

The data presented above prove the natural existence of several isomers of $InsP_6$-related phosphoinositol diphosphates in the vegetative amoebae of *Dictyostelium discoideum*. The cellular concentration of these energy-rich inositol phosphates is about half that of $InsP_6$. It may be speculated that these compounds provide the cells with a source of chemical energy, phosphate, carbohydrate, and metal ions, in one single type of complex which may facilitate the cellular survival in an unfavourable environment.

NMR investigations are in progress, in G.W.M.'s laboratory, in order to clarify the precise positional isomerism of these phosphoinositol diphosphates.

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