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The quantitative spectrum of inositol phosphate metabolites in avian erythrocytes, analysed by proton n.m.r. and h.p.l.c. with direct isomer detection

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The spectrum of inositol phosphate isomers present in avian erythrocytes was investigated in qualitative and quantitative terms. Inositol phosphates were isolated in micromolar quantities from turkey blood by anion-exchange chromatography on Q-Sepharose and subjected to proton n.m.r. and h.p.l.c. analysis. We employed a h.p.l.c. technique with a novel, recently described complexometric post-column detection system, called 'metal-dye detection' [Mayr (1988) Biochem. J. **254**, 585–591], which enabled us to identify non-radioactively labelled inositol phosphate isomers and to determine their masses. The results indicate that avian erythrocytes contain the same inositol phosphate isomers as mammalian cells. Denoted by the 'lowest-locant rule' [NC-IUB Recommendations (1988) Biochem. J. **258**, 1–2] irrespective of true enantiomerism, these are Ins(1,4)P_2, Ins(1,6)P_2, Ins(1,3,4)P_3, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, Ins(1,4,5,6)P_4, Ins(1,3,4,5,6)P_5, and InsP_6. Furthermore, we identified two inositol trisphosphate isomers hitherto not described for mammalian cells, namely Ins(1,5,6)P_3 and Ins(2,4,5)P_3. The possible position of these two isomers in inositol phosphate metabolism and implications resulting from absolute abundances of inositol phosphates are discussed.

INTRODUCTION

High amounts of Ins(1,3,4,5,6)P_5 in avian erythrocytes (Johnson & Tate, 1969; Mayr & Dietrich, 1987) indicate a well-developed synthesis of this inositol pentakisphosphate isomer. As InsP_5, in most cases obviously the Ins(1,3,4,5,6)P_5 isomer, is also synthesized in many mammalian cell types (Balla *et al.*, 1987; Tilly *et al.*, 1987; Vallejo *et al.*, 1987; Szwergold *et al.*, 1987; Dean & Moyer, 1988; Mayr, 1988) the question arises whether similar metabolism of inositol phosphates occurs in cells of both vertebrate classes. Recent findings indicate the same spectrum of inositol phosphate isomers for both avian and mammalian cells. Stephens *et al.* (1988*a*) demonstrated the presence of L-Ins(1,4,5,6)P_4 in avian erythrocytes and mouse bone macrophages. Morris *et al.* (1987) identified a soluble and membrane-bound activity of D-Ins(1,4,5)P_3 3-kinase in turkey erythrocytes and the product of this reaction, D-Ins(1,3,4,5)P_4, was shown by Stephens *et al.* (1988*a*) to be present in [^{3}H]inositol-labelled chick erythrocytes. In mammalian tissues a dephosphorylation product of D-Ins(1,3,4,5)P_4, namely D-Ins(1,3,4)P_3, can be phosphorylated to another tetrakisphosphate isomer, Ins(1,3,4,6)P_4 (Balla *et al.*, 1987; Shears *et al.*, 1987*b*; Hansen *et al.*, 1988). Stephens *et al.* (1988*b*) showed that, in rat brain homogenates, Ins(1,3,4,6)P_4 can be a precursor of Ins(1,3,4,5,6)P_5. A similar kind of phosphorylation to Ins(1,3,4,5,6)P_5 might be present in avian erythrocytes, as the same authors were able to demonstrate the existence of Ins(1,3,4,6)P_4 in this cell type.

In most cases inositol phosphates in avian erythrocytes were examined by means of co-chromatography with known standards, or chemically by periodate oxidation and dephosphorylation. Additional proof of the configuration of inositol phosphates can be achieved by applying n.m.r. techniques. In a former work (Mayr & Dietrich, 1987) we described the identification of Ins(1,4,5,6)P_4 in turkey erythrocytes with the help of n.m.r. Here we report the identification of Ins(1,4)P_2, Ins(1,6)P_2, Ins(1,3,4)P_3, Ins(1,4,5)P_4 Ins(1,3,4,5)P_4, and Ins(1,3,4,6)P_4 in turkey erythrocytes by ^{1}H-n.m.r. Furthermore, we identified two hitherto undetected inositol trisphosphate isomers, namely Ins(1,5,6)P_3 and Ins(2,4,5)P_3. This required the development of a highly resolving chromatographic system to separate sufficient amounts of inositol phosphates extracted from avian erythrocytes. Application of a recently developed h.p.l.c. technique (Mayr, 1988), suitable for direct detection of unlabelled inositol phosphates, allowed a precise mass determination of most isomers in avian erythrocytes. As in the present study all inositol bis-, tris-, and tetrakis-

Abbreviations used: InsP_1, InsP_2, InsP_3, InsP_4, InsP_5, and InsP_6 are *myo*-inositol mono-, bis-, tris-, tetrakis-, pentakis-, and hexakis-phosphate, with locants designated in parentheses where appropriate; without a prefix, isomers are symmetric, e.g. Ins(1,3,4,6)P_4, or enantiometric structures are unassigned, e.g. Ins(1,6)P_2 may be L-Ins(1,6)P_2 {= D-Ins(3,4,)P_2, applying the relaxation of the 'lowest-locant rule' [NC-IUB Recommendations (1988) Biochem. J. **258**, 1–2]} , D-Ins(1,6)P_2 or a racemic mixture of both; a 'D' or 'L' prefix is only added when specific enantiomers are to be denoted; Ins(1:2cyc,4,5)P_3, D-*myo*-inositol 1:2-cyclic,4,5-trisphosphate; PAR, 4-(2-pyridylazo)resorcinol; m.d.d., metal-dye detection; COSY, correlation spectroscopy.

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phosphate isomers present in avian blood in above 10^{-7} M concentration have been identified, we anticipate that major pathways of formation and degradation of Ins(1,3,4,5,6)P_5 should involve these metabolites.

MATERIALS AND METHODS

Polyphosphates and D-glucose 6-phosphate (analytical grade) were obtained from Sigma (St. Louis, MO, U.S.A.). 4-(2-Pyridylazo)resorcinol (PAR) was from Serva (Heidelberg, Germany). All other materials, chemicals and inositol phosphate standards were described by Mayr (1988).

Isolation of inositol phosphates from turkey erythrocytes

Inositol phosphates were extracted by a slight modification of the procedure described by Mayr & Dietrich (1987). All steps were performed at 4 °C. A 3.3 litre portion of turkey blood, freshly obtained from a turkey slaughterhouse, was mixed with 3.3 litres of ice-cold 1 M-perchloric acid. The precipitate was removed by centrifugation (20 min at 10000 *g*). The supernatant was adjusted to pH 5 by adding KOH and afterwards filtered to remove the precipitated perchlorate. The filtrate, termed 'crude inositol phosphate extract' in the following, was freeze-dried. After the dry material had been resuspended in 700 ml of ice-cold water the pH was again adjusted to 5 and the remaining perchlorate removed by filtration. From this solution nucleotides were removed by charcoal treatment (2 × 20 g of Norit A). The extract was cleared from charcoal by centrifugation and filtration. To decrease conductivity the extract was once more freeze-dried, and, after resuspending the residue in 200 ml of water, the remaining perchlorate was removed as described above. The resulting filtrate was diluted with water to a conductivity of 3 mS and the pH adjusted to 8.

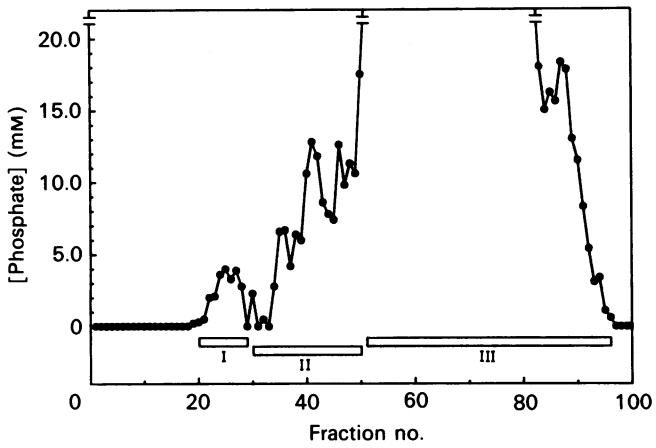

Fig. 1. Separation of inositol phosphates from turkey blood on a short Q-Sepharose column

An extract of 3.3 litres of turkey blood was loaded on to a short Q-Sepharose column and eluted with a gradient of ammonium formate as described in the Materials and methods section. Pools were selected in order to separate fractions containing inositol bis-, tris- and tetrakis-phosphates (pool II) from fractions with orthophosphate (pool I) and Ins(1,3,4,5,6)P_5 (pool III). Pooled fractions are indicated by bars. The material of pool II was re-chromatographed (see the Materials and methods section and Fig. 2).

This solution was applied, at a flow rate of 800 ml/h, to a column of Q-Sepharose (formate form, column dimensions 5.5 cm × 10.5 cm). Inositol phosphates were eluted with a linear gradient of 2 litres of ammonium formate (0–1.2 M)/0.1 M-formic acid with a flow rate of 800 ml/h. Fractions (20 ml each) were collected and assayed for organic and inorganic phosphate (Bartlett, 1959; Lanzetta *et al.*, 1979). This chromatographic procedure separated orthophosphate and InsP_5 from all other phosphate-containing fractions, which were collected by an appropriate pooling (see Fig. 1). H.p.l.c.–m.d.d. analysis of pools I, II and III, performed as described below, confirmed that only pool II contained inositol bis-, tris-, and tetrakis-phosphates (results not shown). Pooled fractions were freeze-dried several times to remove ammonium formate. The remaining material was dissolved in water and diluted to a conductivity of 2 mS. After adjusting the pH to 7, the solution was applied at a flow rate of 200 ml/h to a column of Q-Sepharose (chloride form, column dimensions 1.5 cm × 165 cm). A linear gradient of 3 litres of 0–0.4 M-HCl was applied to elute inositol phosphates. Fractions (10 ml) were rapidly assayed for organic and inorganic phosphate and for absorbance at 260 nm. The resulting chromatogram is shown in Fig. 2. Peak fractions, pooled as shown in Fig. 2, were immediately diluted with water and freeze-dried

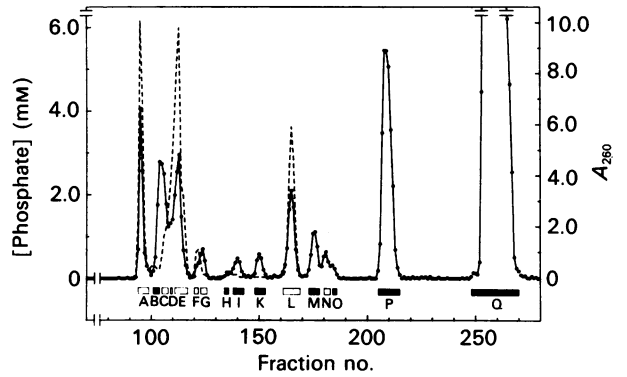

Fig. 2. Separation of inositol phosphates from turkey blood on a long Q-Sepharose column

The material of pool II of the preceding separation step (cf. Fig. 1) was re-chromatographed on a long column of Q-Sepharose as described in the Materials and methods section. Fractions were pooled as indicated by bars and further analysed by h.p.l.c.–m.d.d. and by ^{1}H-n.m.r. (see the Materials and methods and the Results sections for detailed information). Dark bars (■) indicate pools with mainly inositol phosphates. Pools containing mainly compounds other than inositol phosphates are marked by light bars (□). Substances identified are the following [an asterisk (*) indicates assignments resulting from n.m.r. analysis; a question mark (?) demonstrates uncertain assignments]: pool A, P_i + InsP_1 + nucleoside mono- and di-phosphates; pool B, Ins(1,4)P_2* + Ins(1,6)P_2* + glucose 1,6-bisphosphate*; pool C, fructose 1,6-bisphosphate; pool D, nucleoside diphosphate(s); pool E, unidentified nucleotide + UDP + PP_i; pool F, ATP; pool G, glycerate 2,3-bisphosphate; pool H, Ins(1,3,4)P_3*; pool I, mainly Ins(1,4,5)P_3* + Ins(2,4,5)P_3* with minor amounts of Ins(1,5,6)P_3 + unknown; pool K, Ins(1,5,6)P_3*; pool L, Ins(4,5,6)P_3? + UTP; pool M, Ins(1,3,4,6)P_4*; pool N, *P*-X-*PP*?; pool O, Ins(1,3,4,5)P_4*; pool P, Ins(1,4,5,6)P_4; pool Q, Ins(1,3,4,5,6)P_5.

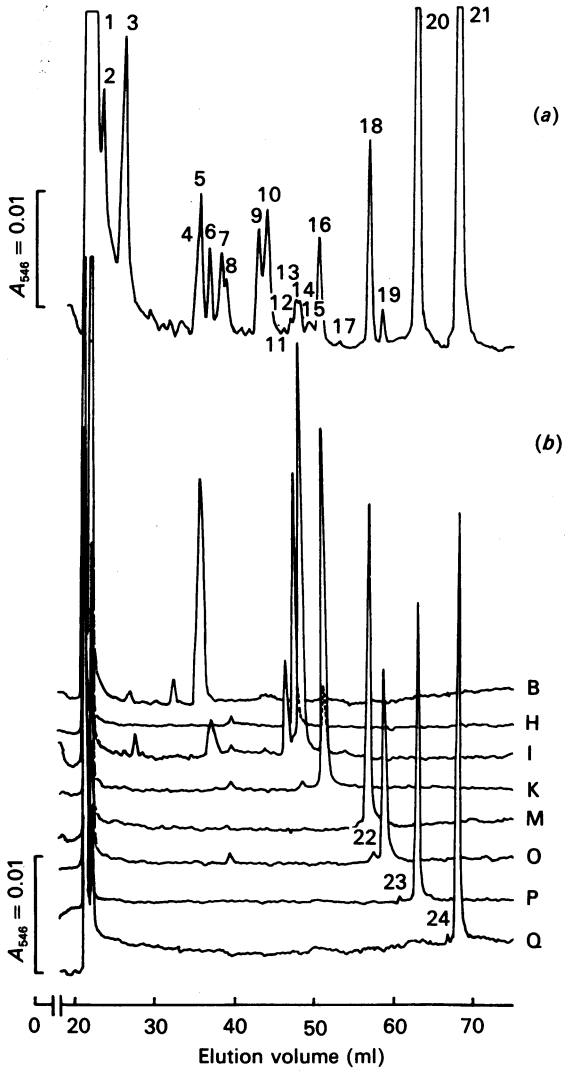

Fig. 3. H.p.l.c. analysis of inositol phosphates from turkey blood

Separation and detection of inositol phosphates by h.p.l.c.–m.d.d. was performed as described in the Materials and methods section. (*a*) Material obtained after first chromatography on Q-Sepharose (pool II in Fig. 1). An aliquot containing 100 nmol of total phosphate was injected. (*b*) Inositol phosphate pools marked in Fig. 2 by dark bars. Capital letters indicate analysed pools, which are named as in Fig. 2. Aliquots (about 1 nmol each) of inositol phosphates were injected. Peaks were assigned to specific compounds by co-chromatography with commercially available standards or with preparatively isolated inositol phosphates, which were identified by n.m.r. analysis (a question mark indicates uncertain assignments). 1, P_i+InsP_1+nucleoside mono- and di-phosphates+fluoride+acetate; 2, unknown+ADP; 3, unknown+ATP; 4, GTP; 5, Ins(1,4)P_2+Ins(1,6)P_2+glucose 1,6-bisphosphate; 6, fructose 1,6-bisphosphate; 7, UDP; 8, PP_i; 9, glycerate 2,3-bisphosphate; 10, UTP; 11, unknown; 12, Ins(1,3,4)P_3; 13, Ins(1,4,5)P_3; 14, Ins(2,4,5)P_3; 15, *P*-X-*PP*?; 16, Ins(1,5,6)P_3; 17, Ins(4,5,6)P_3?; 18, Ins(1,3,4,6)P_4; 19, Ins(1,3,4,5)P_4; 20, Ins(1,4,5,6)P_4; 21, Ins(1,3,4,5,6)P_5; 22, Ins(1,2,4,5)P_4; 23, Ins(2,4,5,6)P_4; 24, Ins(1,2,4,5,6)P_5.

to remove the HCl. Aliquots from these pools were analysed by h.p.l.c. as described below. The results are demonstrated in Fig. 3 (*b*). An aliquot of the solution applied to the long Q-Sepharose column was also subjected to h.p.l.c. analysis. This elution profile is shown in Fig. 3 (*a*).

N.m.r. measurements

Freeze-dried inositol phosphates were dissolved in 1 ml of ^2H_2O and neutralized with $N^2H_4O^2H$. Samples were further prepared for n.m.r. as described by Mayr & Dietrich (1987). Pool I needed an additional purification step to remove interfering impurities; the freeze-dried material was diluted with 3 ml of water and loaded on to a small column of Q-Sepharose (1 ml, formate form). Inositol phosphates were eluted with 7 ml of 0.4 M-ammonium formate/0.03 M-formic acid. After removing ammonium formate by freeze-drying, pool I was processed for n.m.r. as described above.

All samples were adjusted to a p^{2}H value of 6.0 and measured at 300 K. The sample volume was 700 μl, with 0.5 μl acetone as internal standard. ^{1}H-n.m.r. spectra were collected with a Bruker AM-400 spectrometer operating at 400.13 MHz. For one-dimensional-spectrum experiments pulses of $\pi/2$ and relaxation delays of 11 to 22 s between each acquisition were employed. The two-dimensional spectrum was obtained by using a normal correlation spectroscopy (COSY) [J. Jeener, unpublished work (but presented verbally at the Ampere International Summer School II at Basko Polje, Yugoslavia, in 1971)] with a pulse sequence of $\pi/2-\tau-\pi/4-$acq. By this so-called 'COSY-45 technique', diagonal signals are reduced in intensity. The spectrum was recorded between 1.5 and 5.5 p.p.m. with a spectral width of 1602.5 Hz. The two-dimensional-spectrum experiments were set up assuming an H–H coupling constant of 7.5 Hz, and the experimental parameters were adjusted accordingly. For recording, a 256 × 1024 word data matrix was employed, which was zero-filled before Fourier-transformation to obtain a 1024 × 2048 spectral data matrix. This resulted in a resolution of 0.78 Hz/point.

Chemical shifts are given relative to that of tetramethylsilane (TMS) which was assigned to 0 p.p.m., but actually acetone was employed as an internal standard. Its chemical shift in ^2H_2O, 2.225 p.p.m., was used for chemical-shift evaluation. [Note: in a former paper (Mayr & Dietrich, 1987) a chemical shift of 2.040 was used for acetone. For comparison with data given here, chemical shifts from that paper must be increased by 0.185 p.p.m.]

H.p.l.c.–m.d.d.

H.p.l.c. analyses, using metal-dye detection (m.d.d.), were in general performed as described by Mayr (1988). The column system consisted of a guard column (0.5 cm × 5 cm) and a main column (0.5 cm × 20 cm) packed with Mono Q. The gradient was mixed with a flow rate of 1 ml/min using eluent A (14 μM-YCl_3/200 μM-HCl) and B (14 μM-YCl_3/0.5 M-HCl) and had the following characteristics: 0 min, 0% B; 11 min, 0% B; 11.1 min, 4% B; 21 min, 8% B; 30 min, 14.4% B; 43 min, 28% B; 51 min, 44% B; 55 min, 60% B; 60 min, 90% B; 63 min 100% B; 70 min, 100% B. The post-column-added dye solution was pumped at a flow rate of 0.55 ml/min. It contained a 200 μM concentration of the metal-binding indicator PAR and 1.4 M-triethanolamine at a pH of 8.55. Before the eluate was mixed with the dye solution it passed through a u.v. monitor (LKB 2151) set to 260 nm.

All samples loaded on to the column were diluted to

2 ml with 2.5 mM-sodium acetate/1 mM-sodium fluoride, pH 5. For quantification of inositol phosphates from turkey blood by h.p.l.c., the extract was analysed before and after charcoal treatment. Because the concentrations of individual inositol phosphates were very variable, different volumes of extract were injected to achieve peak areas within the range of the calibration curve (see below). In the first case, i.e. analyses before charcoal treatment, 1.6, 16, 48, and 240 μl of 'crude inositol phosphate extract' (see above), corresponding to 1, 10, 30, and 150 μl of turkey blood, were loaded on to the h.p.l.c. column without further purification. For charcoal treatment, 600 μl of this extract were diluted to 1.5 ml with 2.5 mM-NaF/0.5 mM-EDTA at pH 5. This solution was twice treated with 60 μl of a 20% (w/v) suspension of Norit A as described by Mayr (1988). After centrifugation, charcoal pellets were washed with 0.6 ml of 0.1 mM-NaCl/2.5 mM-NaF/0.5 mM-EDTA at pH 5. Supernatant and wash solution were combined, diluted to 45 ml with water, and loaded on to a small column of Q-Sepharose (1 ml; chloride form). After washing the column with 12 ml of 2 mM-HCl, inositol phosphates were eluted with 10 ml of 0.54 M-HCl. The eluate was immediately frozen and freeze-dried to remove the HCl. The dried sample was dissolved in 3 ml of 2.5 M-sodium acetate/1 mM-NaF at pH 5. Aliquots [8, 80, 240 and 1200 μl (corresponding to 1, 10, 30 and 150 μl of turkey blood)] were injected for h.p.l.c. analysis. For calibration, a standard of partly hydrolysed phytic acid was employed. The conditions of hydrolysis were described by Phillippy *et al.* (1987). To determine the composition of this standard, an aliquot (approx. 3 mmol of total phosphorus) was separated on a 165 cm column of Q-Sepharose exactly as described above. The masses of individual inositol phosphates eluted from this column were determined by a phosphate test, their structure by n.m.r. investigation (results not shown here). The standard contained a broad spectrum of inositol phosphates, including most of the inositol phosphate isomers isolated from turkey blood (see below). Various amounts of this standard were injected for h.p.l.c. analysis. The resulting peak integrals were utilized to calculate calibration curves by non-linear regression.

RESULTS

Preparative isolation of inositol phosphates from turkey blood

Avian blood represents an excellent source of higher phosphorylated inositol phosphates from mainly one cell type, the erythrocyte, which represents more than 95% of the total cell volume. H.p.l.c. analysis demonstrated that, in serum, inositol phosphates are absent (results not shown). However, use of large-diameter columns of Dowex anion-exchange resins and standard ammonium formate or HCl gradients never revealed satisfying separations of inositol phosphate isomers from large extract volumes. Furthermore, the resolving efficiency of the columns was reduced by large amounts of Ins(1,3,4,5,6)P_5 and nucleotides present in the extracts.

A dramatic improvement was achieved by the following changes. Firstly, we removed most of the nucleotides by adsorption to charcoal. Secondly, Q-Sepharose was used instead of Dowex 1X2. This low-capacity anion-exchanger has mechanical stability similar to that of Dowex 1X2, but requires less than half the eluent ionic strength as the former resin for elution of identical inositol phosphates. And finally, by appropriate pooling after chromatography on a short column of Q-Sepharose (see Fig. 1 and the Materials and methods section) the excessive amounts of P_i and Ins(1,3,4,5,6)P_5 were largely eliminated. The use of ammonium formate as an eluent prevented the pooled material from acid migration during freeze-drying which might take place when HCl was used. After removal of the ammonium formate, this material could then be effectively chromatographed on a long column of Q-Sepharose, which was developed with a shallow gradient of HCl (see the Materials and methods section). HCl was used because it has a much better isomer-resolving efficiency for inositol phosphates containing three or more phosphates than ammonium formate (Mayr, 1988). Acidic conditions as employed during extraction and chromatography of inositol phosphates can give rise to phosphate migration between *cis*-orientated hydroxy groups. In order to investigate the extent of phosphate migration, several known inositol phosphate isomers [Ins(1,4,5)P_3, Ins(2,4,5)P_3, Ins(1,5,6)P_3, Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5] were subjected to similar extraction and chromatographic conditions to blood samples. H.p.l.c. analysis could not reveal any phosphate migration when inositol phosphates were left at room temperature for 6 h in 0.5 M-$HClO_4$. Only after freeze-drying of inositol phosphates from HCl (as eluted Q-Sepharose) was phosphate migration detectable; in general about 2–13% of compounds named above were transformed to corresponding C-2 or C-1 phosphorylated isomers. The extent of phosphate migration never exceeded 19% and in no case did dephosphorylation occur. Therefore care was taken to accelerate freeze-drying by using a thin frozen film. With these precautions, only a minimal phosphate migration could be detected by h.p.l.c. analysis of isolated inositol phosphates (see Fig. 3*b* and below).

Assignment of inositol phosphate isomers by h.p.l.c.–m.d.d.

Aliquots of the separated peaks shown in Fig. 2 were subjected to h.p.l.c. on a column of Mono Q employing m.d.d. Peaks D, F, and L were identified, by their u.v. absorption and their co-migration with known standards (results not shown), as mainly UDP, ATP and UTP respectively. The phosphate-containing compound N, eluted within the range of inositol tetrakisphosphates, was apparently neither a nucleotide nor an inositol phosphate. It had no u.v. absorbance, was extremely acid-labile and exhibited no sugar- or inositol-phosphate-specific resonances when subjected to ^{1}H n.m.r. spectroscopy (results not shown). When analysed by h.p.l.c. after freeze-drying, compounds were found that co-chromatographed with orthophosphate, pyrophosphate and tripolyphosphate (results not shown). But obviously the latter two compounds were not identical with these standards; the compounds that were co-eluted with tripolyphosphate and pyrophosphate on Mono Q were not co-eluted with these standards on Q-Sepharose. After boiling in acid (10 min at 100 °C in 0.5 M-$HClO_4$) the compound eluted with tripolyphosphate on Mono Q completely disappeared, but the substance which co-chromatographed with pyrophosphate remained stable. All these data direct one towards a substance of the structure *P*-X-*P*-*P*, which under acidic conditions could be degraded to acid-stable *P*-X-*P* and P_i. All other peaks

which are marked in Fig. 2 by light bars were identified by co-chromatography with known standards (see the legend to Fig. 2). The peaks marked by dark bars were isomers or mixtures of isomers of InsP_2, InsP_3, InsP_4 and InsP_5. Their h.p.l.c. elution profiles are shown in Fig. 3(*b*). For comparison, Fig. 3(*a*) shows the analysis of unfractionated inositol phosphate of pool II (cf. Fig. 2). The material of pool B was eluted together with known Ins(1,4)P_2, but the broad peak indicates the presence of more than one InsP_2 isomer and/or other substances with similar chromatographic properties, e.g. hexose bisphosphates. The material separated in peak H and K co-chromatographed with Ins(1,3,4)P_3 and Ins(1,5,6)P_3 respectively, which are present in the inositol phosphate standard mixture (see the Materials and methods section). The main substance in pool I co-eluted together with known Ins(1,4,5)P_3. N.m.r. studies (below) revealed the presence of both Ins(1,4,5)P_3 and Ins(2,4,5)P_3 in pool I, and the separation shown in Fig. 3(*a*) also indicated two distinct compounds (marked '14' and '15') in the Ins(1,4,5)P_3 region. After isolation, however, these two isomers were no longer separated. Since the addition of extract to pool I material restored separation, unknown components in the extract seemed to change slightly the elution behaviour of Ins(2,4,5)P_3. The substance that was eluted immediately before Ins(1,4,5)P_3 [and Ins(1,3,4)P_3 in pool H] could not be identified with certainty. It was eluted at a position where, in the standard mixture of inositol phosphates (see the Materials and methods section), Ins(2,4,6)P_3 and Ins(1,3,5)P_3 were identified by n.m.r. A further minor compound in pool I chromatographed together with the isomer present in pool K, namely Ins(1,5,6)P_3. The presence of Ins(1,5,6)P_3 could in fact be explained by phosphate migration in Ins(2,4,5)P_3 which obviously occurred during freeze-drying (see above). Compound M, a putative InsP_4, could not be identified by co-chromatography with available inositol phosphate standards. The remaining peaks, designated O, P and Q, could be assigned by co-chromatography with commercially available standards to the isomers Ins(1,3,4,5)P_4, Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 respectively. The h.p.l.c. chromatograms of these three pools also indicated the presence of small quantities of inositol phosphates phosphorylated at C-2 and which amounted to 1.6–6.9% of the corresponding C-1/C-3-phosphorylated isomers. Their generation can clearly be explained by phosphate migration of the C-1/C-3-phosphorylated parent compounds during freeze-drying (see above). A further investigation of pool L revealed the presence of a small amount of another inositol trisphosphate which co-migrated with Ins(4,5,6)P_3 in the inositol standard mixture (results not shown). The small quantities of this substance permitted no n.m.r. investigation, and therefore identification of this isomer has to remain indefinite.

^{1}H-n.m.r. identification of inositol phosphate isomers

In order to confirm the structures of all inositol bis-, tris- and tetrakis-phosphate isomers present in avian erythrocytes we subjected isolated species except the previously investigated isomer Ins(1,4,5,6)P_4 (Mayr & Dietrich, 1987) to high-resolution ^{1}H-n.m.r. spectroscopic analysis. The results of this analysis are shown in Figs. 4–7.

In Figs. 4(*a*)–4(*c*) the identification of inositol bisphosphates and contaminating hexose phosphates present in pool B (cf. Figs. 2 and 3) is demonstrated. The one-dimensional ^{1}H-n.m.r. spectrum in Fig. 4(*a*) indicates the presence of several compounds. The resonances at between 4.6 and 5.5 p.p.m. clearly can be assigned to anomeric sugar protons which, in a non-phosphorylated state of the semiacetalic hydroxy group, show the observed doublet structures. Only a very small fraction of these sugar phosphates appears to be phosphorylated at this group, since the resulting duplicated doublet structures are practically absent (only one at about 5.4 p.p.m. is evident). But during chromatographic separation on Q-Sepharose all of these sugar phosphates must have been doubly phosphorylated, as only in this instance are they eluted from anion-exchangers like inositol bisphosphates. Apparently most of these sugar bisphosphates have lost their acid-labile acetalic phosphate groups during the subsequent freeze-drying of the pooled fractions, owing to the presence of HCl (the eluent used). A re-chromatography of the material by ammonium formate gradient elution confirmed that now, in addition to a peak containing inositol bisphosphates, an earlier-eluted peak, containing phosphate and phosphomonoesters, was present. An inspection of a corresponding one-dimensional ^{1}H-n.m.r. spectrum of glucose 6-phosphate (shown in Fig. 4*b*) shows that most of the sugar phosphate material present in pool B belongs to the two anomers of glucose 6-phosphate, the β-anomer (corresponding resonances marked 'C' in the spectrum) and the α-anomer (marked 'D'), which is about half as abundant as the β-anomer. As deduced from the integrals of the resonances in Fig. 4(*a*), D-glucose 1,6-bisphosphate must have comprised about 42% of the total content of phospho compounds in pool B. Other originally doubly phosphorylated sugars in this pool amount to no more than 11%. Among them β-mannose 1,6-bisphosphate appears to be the most abundant (results not shown). The remaining 47% of doubly phosphorylated compounds could be assigned to inositol bisphosphates. Only two isomers were found and no inositol monophosphate, owing to the much greater stability of these substances in diluted HCl compared with hexose bisphosphates (Fasman, 1975). These two isomers could be clearly assigned, by the results of the ^{1}H–^{1}H COSY experiment demonstrated in Fig. 4(*c*), to Ins(1,6)P_2 and Ins(1,4)P_2. the assignments and connectivities deduced from off-diagonal resonances in Fig. 4(*c*) have been marked 'A' for protons of Ins(1,6)P_2, 'B' for protons of Ins(1,4)P_2, 'C' or 'D' for protons of the two anomers of glucose 6-phosphate. The same capital letters denote the coupling patterns of the corresponding resonances depicted in the one-dimensional spectrum of Figs. 4(*a*) and 4(*b*). Assignments to the two inositol bisphosphates are marked mainly above, and those to D-glucose 6-phosphate mainly below, the diagonal correlation line. Both from the spectrum in Fig. 4(*a*) and from this COSY experiment it is quite evident that only two resonances corresponding to inositol protons H-2 are present. Both protons are without a vicinal phosphorus atom, which is shown by the characteristic triplets with $^3J_{HCCH}$ of about 2.5 Hz (Mayr & Dietrich, 1987). The lack of additional H-2 specific resonances indicates the absence of any other inositol bisphosphate, e.g. Ins(1,3)P_2, or inositol bisphosphates bearing phosphate at position 2. Both inositol bisphosphates have a triplet resonance at about 3.5 p.p.m. characteristic of

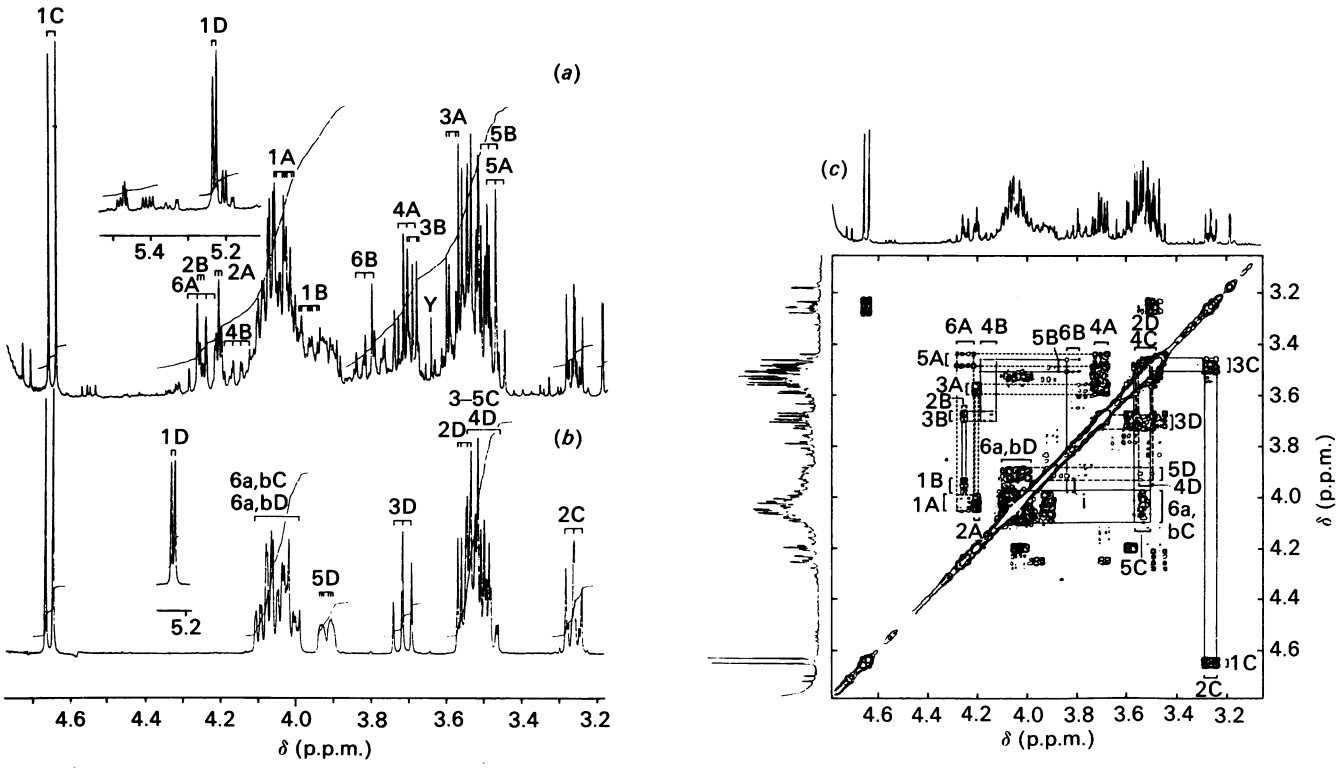

Fig. 4. ^{1}H-n.m.r. identification of inositol bisphosphates from turkey erythrocytes

(*a*) One-dimensional spectrum of the material of pool B (Fig. 3). For sample preparation and acquisition conditions see the Materials and methods section. Material amounting 42 μmol of phosphate was employed. Besides the region upfield from the $^1H^2HO$ resonance at about 4.8 p.p.m., the range downfield from $^1H^2HO$ showing anomeric sugar resonances is depicted in the inset. Resonances are marked according to their splitting characteristics, and, above, a number denotes the proton of the inositol ring and a capital letter the compound: A, Ins(1,6)P_2; B, Ins(1,4)P_2. (*b*) One-dimensional spectrum of glucose-6-phosphate; 50 μmol of substance was analysed. The spectral window was the same as in (*a*); the inset shows the resonance corresponding to the α-anomeric H-1. Resonances are marked as in (*a*): C, β-glucose 6-phosphate; D, α-glucose 6-phosphate. (*c*) COSY experiment performed with the same material. Alongside both axes the one-dimensional spectrum from (*a*) is plotted. For sake of more clarity, off-diagonal resonances are marked as in (*a*) by proton numbers and capital, together with a square bracket open towards the axes indicating the chemical shift of the resonance. Cross correlations of compounds A and B are marked mainly in the upper half square, those of compounds C and D in the lower one. Connectivities between neighboured protons are indicated by broken (A,D) and unbroken (B, C) lines. 6a,bC and 6a,bD denote the complex resonances of the two protons at position C-6 of β-glucose-6-phosphate and α-glucose 6-phosphate respectively. The H–1 : H–6 cross-correlation of B is only partially visible and marked 'i'. Crowding in the 3.55 and 4.05 p.p.m. regions prevented complete assignments of glucose 6-phosphate resonances. The doublet visible at 3.18 p.p.m. is due to an unidentified contaminant.

'non-phosphorylated' H-5 of the inositol, and both contain one vicinal phosphorus atom at either H-1 or H-3 (duplicated triplet-like doublet of a doublet of a doublet, with $^3J_{HCCH}$ values of about 9 and 2.5 Hz and a $^3J_{HCOP}$ of about 9 Hz) and at either H-4 or H-6 (quartet-like duplicated triplet, $^3J_{HCCH}$ and $^3J_{HCOP}$ values of about 9 Hz; cf. Mayr & Dietrich, 1987; Cerdan *et al.*, 1986). As a result of this phosphate distribution only two isomers are possible : Ins(1,6)P_2 and Ins(1,4)P_2. Since in Ins(1,6)P_2 the two neighbouring protons with vicinal phosphorus nuclei must be more deshielded than the corresponding non-neighbouring protons in Ins(1,4)P_2, the compound with the more downfield-shifted corresponding resonances must be Ins(1,6)P_2. This compound can be estimated from the integrals of the uncrowded resonances H-2 and H-4/H-6 (between 4.1 and 4.3 p.p.m.) in Fig. 4(*a*) to be about 3-fold as abundant as Ins(1,4)P_2. Another abundant bisphosphorylated sugar, fructose 1,6-bisphosphate, was completely absent from pool B. This is in agreement with the h.p.l.c. data (see Figs. 3*a* and 3*b*) showing, in pool B, no fructose 1,6-bisphosphate, which was identified in the subsequent pool, C. The elution behaviour of InsP_2 and hexose bisphosphates on Q-Sepharose thus appears to be very similar to that on the Mono-Q material employed for h.p.l.c.

Fig. 5(*a*) shows the ^{1}H-n.m.r. spectrum of the material from pool H. Although several peaks apparently resulting from contaminants in the eluent are present (two of them are marked 'Y' and 'Z' in the spectrum), only six groups of resonances which are characteristic for an inositol phosphate can be detected, indicating that peak H contains only one phosphorylated inositol which is magnetically asymmetric. The resonance at 4.41 p.p.m. is characteristic for a proton at position 2 of the inositol ring without a phosphate group adjacent to C-2 (see above). There are two more resonances typical for inositol protons located at carbon atoms carrying no phosphoester group, namely those at 3.84 and 3.55 p.p.m. The former one can clearly be identified as a triplet with $^3J_{HCCH}$ of about 9 Hz. The identification of the latter very similar triplet is somewhat obscured by several solvent

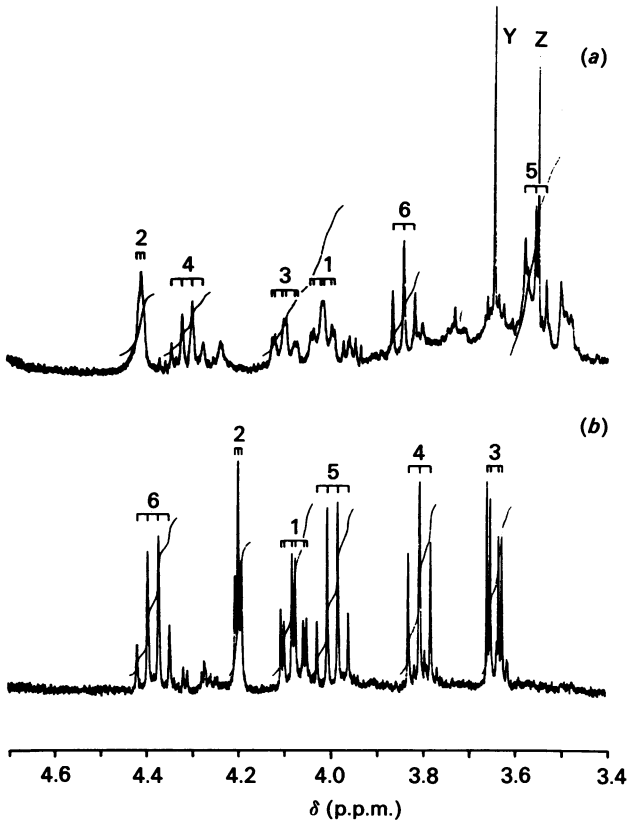

Fig. 5. Proton-n.m.r. identification of inositol trisphosphates isolated from turkey erythrocytes

(*a*) Spectrum of 0.53 μmol of Ins(1,3,4)P_3 present in pool. H (Fig. 3*b*). Resonances are marked according to the inositol-ring numbering as described in Fig. 4. Y and Z denote solvent impurities also found in other pools from the Q-Sepharose chromatography. Unresolved resonances between 3.4 and 3.8 p.p.m., at 3.95 p.p.m. and at 4.25 p.p.m. are also detectable in the spectra recorded from pools L and M (see Fig. 7) and are also likely due to solvent impurities or material bleeding from the Q-Sepharose. (*b*) Spectrum of 3.3 μmol of Ins(1,5,6)P_3 isolated in pool K (Fig. 3*b*).

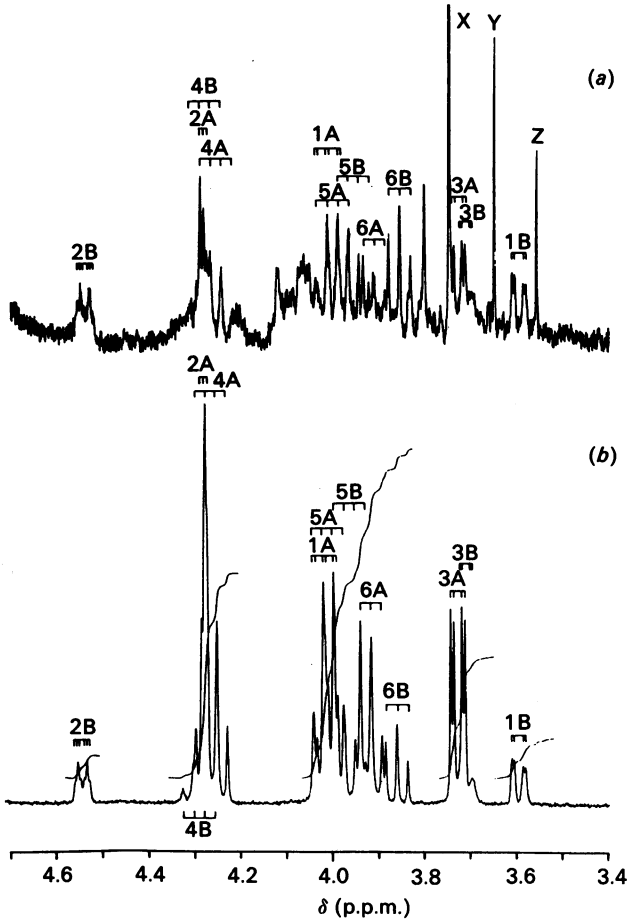

Fig. 6. ^{1}H-n.m.r. identification of inositol trisphosphates from turkey erythrocytes

(*a*) Spectrum of the material contained in pool I (Fig. 3*b*), amounting to 5.6 μmol of phosphate. A denotes Ins(1,4,5)P_3 B denotes Ins(2,4,5)P_3 and X, Y and Z are solvent contaminants. (*b*) Reference spectrum collected from a mixture of 15.2 μmol of pure Ins(1,4,5)P_3 and 4.8 μmol of Ins(2,4,5)P_3.

contaminant resonances (especially that designated 'Z') but is still clearly evident. Both resonances are characteristic for inositol protons with two neighbouring *trans*-orientated protons, i.e. for protons 4, 5, or 6 (see above and Mayr & Dietrich, 1987). By its typical chemical shift the latter resonance can be assigned to H-5. The remainder can be either H-4 or H-6. A comparison of the two resonance patterns at 4.02 and 4.10 p.p.m. with those of other phosphorylated inositols shows that they can only arise from protons 1 or 3 with phosphate groups adjacent. The resulting vicinal H-C-O-P spin–spin coupling of about 9 Hz causes the doublet of a doublet structure (with coupling constants of about 3 and 9 Hz) to be split into the observed duplicated pseudotriplet (see above). Therefore, the inositol phosphate present here must be phosphorylated both at C-1 and C-3. The remaining resonance at 4.31 p.p.m. exhibits a quartet-like duplicated triplet structure characteristic for H-4 or H-6. The resulting inositol trisphosphate thus has the structure Ins(1,3,4)P_3. The final proton assignment is as indicated in Fig. 5(*a*) and in Table 1 (below) according to this nomenclature. Among the two resonances at 4.02 and 4.11 p.p.m. the more-downfield-shifted one must correspond to the proton which is deshielded by a neighbouring phosphate, i.e. H-3.

The ^{1}H-n.m.r. analysis of the material from pool I is shown in Fig. 6(*a*). As with inositol bisphosphates (pool B), this analysis was complicated by the presence of more than one compound, contamination by eluent impurities, and, more compromisingly, by the limited amount of material available for analysis. In spite of the resulting unfavourable signal-to-noise ratio, the identity of the inositol trisphosphate isomers present could be established by comparison with the reference spectrum depicted in Fig. 6(*b*). Here a mixture of the Ins(1,4,5)P_3 and Ins(2,4,5)P_3 present in a ratio of about 3:1 was analysed. The assignment of resonances is based on the same criteria as extensively discussed above and by Mayr & Dietrich (1987). Resonances of inositol protons corresponding to Ins(1,4,5)P_3 and Ins(2,4,5)P_3 are designated 'A' and 'B' respectively in both spectra. From a comparison of the resulting resonances it is self-evident that exactly these two isomers of InsP_3 are present in pool I.

This is in agreement with the h.p.l.c. analytical data from this pool. Both major compounds migrated close together with our elution system and the earlier-eluted peak had exactly the retention time of Ins(1,4,5)P_3. However, from Fig. 6(*a*) a different quantitative ratio of these two isomers than in the reference material is evident. Preferentially from a comparison of resonances '3A' with '1B', '2B' or '6B' an equal mass of both compounds can be roughly estimated. The singlet resonances 'X', 'Y' and 'Z' result from solvent impurities, since they are also present in the spectra of other pools. The unresolved resonances between 4.05 and 4.2 p.p.m. and the singlet resonance at about 3.8 p.p.m., which are specific for this pool, indicate the presence of some other material. This substance should be similarly polyanionic as InsP_3 because of its chromatographic behaviour on Q-Sepharose. Also the h.p.l.c. analysis of pool I has shown some minor peaks (see Fig. 3*b*), one co-eluted with fructose 1,6-bisphosphate, one with Ins(1,3,5)P_3 or Ins(2,4,6)P_3, and one with Ins(1,5,6)P_3. Only the last of these is very likely identical with the corresponding reference substance. This conclusion can be drawn from our finding that, on Q-Sepharose and Mono Q chromatography, all inositol phosphates are eluted in the same order by HCl (cf. also the discussion for pool N above). The low masses of individual compounds and the spectrum quality prohibited structural assignments to these compounds.

The ^{1}H-n.m.r. spectrum of the material from peak K is shown in Figure 5(*b*). As in Fig. 5(*a*), the presence of only one asymmetric inositol phosphate isomer can be deduced from the six resonances characteristic for inositol phosphate protons. In comparison with Fig. 5(*a*), much less contaminating material is present, which is plausible from the much higher concentration of this isomer in the pooled fractions (see Fig. 2). Again a resonance typical for a proton adjacent to a non-phosphorylated C-2 is present at 4.20 p.p.m. (see above). The smaller downfield shift as compared with Ins(1,3,4)P_3 indicates that this proton may not have two neighbouring phosphates. As shown in the spectrum of Ins(1,3,4)P_3, one resonance characteristic for H-4 or H-6 without a vicinal phosphorus is present at 4.00 p.p.m. and one typical for H-4 or H-6 with a vicinal phosphorus at 4.39 p.p.m. (quartet-like duplicated triplet). Unlike in Ins(1,3,4)P_3 only one resonance characteristic for a phosphorylated C-1 or C-3 is present at 4.10 p.p.m. (duplicated triplet-like resonance). The second of these resonances (at 3.64 p.p.m.) shows the typical 'non-phosphorylated' coupling pattern, a duplicated doublet. Finally, the resonance of H-5 at 4.00 p.p.m. is again of the quartet-like structure (see above), which is indicative of a phosphate group at position 5. Two isomerisms have to be considered for this InsP_3 species, Ins(1,4,5)P_3 and Ins(1,5,6)P_3. Since the spectrum of the former substance, measured under identical conditions, is different (see Figs. 6*a* and 6*b*; Mayr & Dietrich, 1987), Ins(1,5,6)P_3 must be the InsP_3 isomer present in peak K. Finer details of the n.m.r. spectrum, such as relative chemical shifts, roof effects of neighbouring protons and also the elution behaviour by h.p.l.c., are in agreement with this structural assignment.

The ^{1}H-n.m.r. spectrum of the inositol tetrakisphosphate corresponding to fraction M is shown in Fig. 7(*b*). Just one isomer is present. From the presence of only three groups of resonances and their proportions of

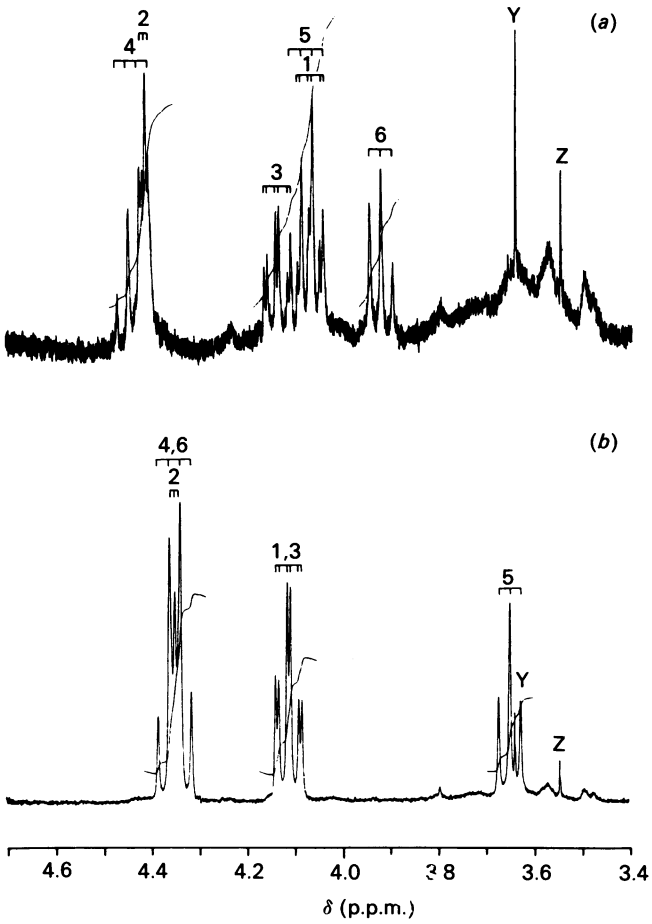

Fig. 7. ^{1}H-n.m.r. identification of inositol tetrakisphosphates from turkey erythrocytes

(*a*) Spectrum of 1.7 μmol of Ins(1,3,4,5)P_4 isolated in pool M (Fig. 3*b*). For Figure notes, see the legends to Figs. 4 and 5. (*b*) Spectrum of 6.2 μmol of Ins(1,3,4,6)P_4 isolated in pool K (Fig. 3*b*).

3 (4.35 p.p.m.):2 (4.12 p.p.m.):1 (3.66 p.p.m.), a symmetric structure for the inositol tetrakisphosphate must be assumed. The triplet at 3.66 p.p.m. with a large $^3J_{HCCH}$ of about 9 Hz can be assigned to H-5 (see the discussion above). The resonance at 4.12 p.p.m. can be assigned by its typical spin–spin coupling pattern (compare H-1 of the spectrum in Fig. 5*b*) to H-1 and, owing to symmetry requirements and the duplicated integral, to H-3, which both must have a vicinal phosphorus. The remaining resonance is composed of a quartet-like resonance characteristic for H-4 and H-6 with vicinal phosphorus and of a triplet with $^3J_{HCCH}$ of about 2.5 Hz that is located just in the centre of the former quartet and can be assigned to H-2. Obviously, this substance is Ins(1,3,4,6)P_4 which, deduced from its peak size in Fig. 2, is the second most abundant InsP_4 isomer in avian erythrocytes.

Peak O could be assigned by its characteristic ^{1}H-n.m.r. spectrum to Ins(1,3,4,5)P_4 (Fig. 7*a*). The resonances are assigned in the Figure, and their coupling patterns are incomplete, and their chemical shifts in very close agreement to those demonstrated in a previous paper (Cerdan *et al.*, 1986). No further inositol phosphate was detectable in this sample

Table 1. Chemical shifts and coupling constants derived from ^{1}H-n.m.r. analysis of inositol phosphates from avian erythrocytes

(*a*) Chemical shifts of individual protons from inositol phosphates. Chemical shifts are expressed relative to that of TMS. Superscripts indicate the observed splitting pattern of resonances: Dd, doublet of a doublet; DDd, doublet of a doublet of a doublet; t (T), triplet; Dt (DT), doublet of a triplet. Capital letters refer to coupling constants of 9–10 Hz, small letters to coupling constants of 2–3 Hz. Approximate chemical shifts are marked by ≈. (*b*) Proton–proton and proton–phosphorus vicinal coupling constants. An asterisk (*) indicates where coupling constants were derived from well-resolved spectra of commercially available inositol phosphates or of inositol phosphates isolated from partly hydrolysed phytic acid (see the Materials and methods section). Values in parentheses are far-distance coupling constants, $^4J_{HCCOP}$. Approximate coupling constants are marked by ≈.

(*a*)

	Proton chemical shift (p.p.m.)					
Isomer	H-I	H-2	H-3	H-4	H-5	H-6
Ins(1,4)P_2	3.97^{DDd}	4.26^{T}	3.70^{Dd}	4.16^{DT}	3.50^{T}	3.82^{T}
Ins(1,6)P_2	4.03^{DDd}	4.21^{T}	3.59^{Dd}	3.71^{T}	3.47^{T}	4.25^{DT}
Ins(1,3,4)P_3	4.02^{DDd}	4.41^{T}	4.10^{DDd}	4.31^{DT}	3.55^{T}	3.84^{T}
Ins(1,4,5)P_3	4.02^{DDd}	4.28^{T}	3.73^{Dd}	4.26^{DT}	4.01^{DT}	3.92^{T}
Ins(2,4,5)P_3	3.60^{Dd}	4.54^{DT}	$\approx 3.71^{Dt}$	$\approx 4.29^{DT}$	3.96^{DT}	3.86^{T}
Ins(1,5,6)P_3	4.08^{DDd}	4.20^{T}	3.64^{Dd}	3.81^{T}	4.00^{DT}	4.39^{DT}
Ins(1,3,4,6)P_4	4.12^{DDd}	4.35^{T}	4.12^{DDd}	4.35^{DT}	3.66^{T}	4.35^{DT}
Ins(1,3,4,5)P_4	4.06^{DDd}	4.41^{T}	4.13^{DDd}	4.43^{DT}	4.07^{DT}	3.92^{T}

(*b*)

	H–H vicinal coupling constant (Hz)						H–P vicinal coupling constant (Hz)					
Isomer	J_{H1-H2}	J_{H2-H3}	J_{H3-H4}	J_{H4-H5}	J_{H5-H6}	J_{H6-H1}	J_{H1-P}	J_{H2-P}	J_{H3-P}	J_{H4-P}	J_{H5-P}	J_{H6-P}
Ins(1,4)P_2*	2.7	2.8	9.9	9.0	9.3	9.7	9.0			8.0		
Ins(1,6)P_2*	2.7	2.9	10.2	9.7	9.7	9.4	≈ 8.8					8.1
Ins(1,3,4)P_3	2.4	2.5	9.4	9.1	9.6	9.1	8.8		9.0	9.1		
Ins(1,4,5)P_3*	2.5	2.8	9.9	9.3	9.3	9.3	9.0			8.9	8.7	
Ins(2,4,5)P_3*	1.8	1.8	9.1	9.9	9.5	9.9	(0.5)	7.9	(1.8)	8.4	8.7	
Ins(1,5,6)P_3	2.8	2.8	10.1	9.1	9.1	9.9	9.9				9.1	9.3
Ins(1,4,5,6)P_4	2.7	2.7	9.7	9.2	9.2	9.7	9.7		9.7	9.0		9.0
Ins(1,3,4,5)P_4*	≈ 2.5	2.6	9.9	9.6	9.4	9.7	9.5		9.9	9.3	9.4	

Table 2. Masses of inositol phosphates from turkey blood

Masses were determined by h.p.l.c.–m.d.d. analysis of 'crude inositol phosphate extract', which was chromatographed before (column A) and after charcoal treatment (column B). Details are described in the Materials and methods section. Column C lists inositol phosphate masses resulting from total phosphate determination of preparatively separated inositol phosphates as shown in Fig. 2. Data in columns A and B (except the InsP_2 data) are means ± S.E.M. for three or four h.p.l.c. analyses (with different amounts injected). Abbreviation: n.d., not determined. Notes: [1]Compound superimposed by nucleotides; [2]data derived from results of n.m.r. analyses (see the text); [3]Ins(1,4,5)P_3 and Ins(2,4,5)P_3 not separated (cf. Fig. 2). [4]InsP_6 and the majority of InsP_5 were removed by appropriate pooling (see Fig. 1). The value given here is the sum of all InsP_5 and InsP_6 masses separated by both chromatographies on Q-Sepharose (cf. the Materials and methods section).

Isomer	Analysis method...	Concn. (μmol/litre of blood)		
		A	B	C
Ins(1,3,4)P_3		0.43 ± 0.07	0.50 ± 0.04	0.42
Ins(1,4,5)P_3		1.05 ± 0.16	1.15 ± 0.10	1.83[3]
Ins(2,4,5)P_3		1.12 ± 0.16	1.24 ± 0.10	
Ins(1,5,6)P_3		1.76 ± 0.04	1.56 ± 0.09	2.01
Ins(4,5,6)P_3?		0.14 ± 0.05	0.13 ± 0.07	n.d.[1]
Ins(1,3,4,6)P_4		5.33 ± 0.35	5.70 ± 0.55	3.12
Ins(1,3,4,5)P_4		1.17 ± 0.11	1.29 ± 0.22	0.60
Ins(1,4,5,6)P_4		26.70 ± 1.40	25.46 ± 0.25	20.43
Ins(1,3,4,5,6)P_5		1142 ± 42	1170 ± 47	860[4]
InsP_6		6.57 ± 0.68	6.93 ± 1.69	

The detailed data of the n.m.r. spectra discussed above are compiled in Table 1.

Quantification of identified inositol phosphates

Masses of inositol phosphates were determined by two techniques: by direct h.p.l.c.–m.d.d. analysis of 'crude inositol phosphate extract' as described in the Materials and methods section and by phosphorus determination of preparatively separated isomers (cf. Fig. 2). The results are summarized in Table 2. Only with charcoal-treated samples (analysis methods B and C) could InsP_2 be quantified. However, a separate determination of both InsP_2 isomers was not possible, since they were not resolved by h.p.l.c. (Fig. 3) or by chromatography on Q-Sepharose (Fig. 2). Glucose 1,6-bisphosphate, although present in the 'crude inositol phosphate extract', did not interfere with the h.p.l.c. analysis of InsP_2, since the freeze-drying from HCl (cf. the Materials and methods section and n.m.r. analysis of pool B) almost completely degraded glucose 1,6-bisphosphate to glucose 6-phosphate and P_i. Approximate masses of individual inositol bisphosphates were calculated on the basis of relative amounts of inositol bisphosphates deduced from n.m.r. analysis (see above). The comparison of h.p.l.c. data (columns A and B of Table 2) shows that charcoal treatment is a reliable tool for eliminating interfering nucleotide material without loosing inositol phosphates. The recovery of preparatively isolated inositol phosphates was estimated by comparing the masses resulting from h.p.l.c. analysis of 'crude inositol phosphate extract' (A and B) with those based on phosphorus determination of isolated inositol phosphates (column C of Table 2). Only inositol phosphates with four or more phosphate groups have been lost to a greater extent.

DISCUSSION

The results obtained in the present work show that all inositol phosphate isomers which are known from mammalian cell types are also present in avian erythrocytes. However, the technique employed here tells us nothing about the enantiomerism of identified structures. Nevertheless, on the basis of existing data on inositol phosphate isomers in avian erythrocytes (Morris *et al.*, 1987; Stephens *et al.*, 1988*a*) we tend to assume D-enantiomers for Ins(1,4)P_2, Ins(1,3,4)P_3, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. This would indicate similar metabolic pathways of inositol phosphates in avian erythrocytes and mammalian cells. Only two InsP_2 isomers, namely Ins(1,4)P_2 and Ins(1,6)P_2, were identified in turkey erythrocytes. Ins(1,3)P_2, which can be formed from D-Ins(1,3,4)P_3 by a 4-phosphatase present in enzyme extracts of several mammalian tissues (Irvine *et al.*, 1987; Bansal *et al.*, 1987) is obviously absent. This might be a special feature of inositol phosphate metabolism of unstimulated avian erythrocytes. As a main source of Ins(1,4)P_2, Ins(1,4,5)P_3 can be considered. For Ins(1,6)P_2 two possible sources exist. It might be formed by dephosphorylation of D-Ins(1,3,4)P_3 (Inhorn *et al.*, 1987; Irvine *et al.*, 1987; Shears *et al.*, 1987*a*,*b*), resulting in L-Ins(1,6)P_2, or by dephosphorylation of Ins(1,5,6)P_2. The latter is demonstrated here to be the most abundant inositol trisphosphate isomer of avian erythrocytes. Surprisingly, this isomer has not yet been described as occurring in mammalian systems. This might show the limits of isotope techniques which cannot detect inositol phosphates with a low specific radioactivity. But Ins(1,5,6)P_3 seems not to be restricted to avian cells, since extracts of mammalian tissues also contain an inositol phosphate which co-migrates with this isomer when analysed by h.p.l.c.–m.d.d. (G. W. Mayr, unpublished work). Although the function of Ins(1,5,6)P_3 is unknown, its structure might suggest a metabolic relationship with L-Ins(1,6)P_2 on the one hand and to the already-identified enantiomers L-Ins(1,4,5,6)P_4 or D-Ins(1,3,4,5)P_4 on the other hand. There arises the possibility of an alternative (de)phosphorylation path-

way for Ins(1,3,4,5)P_4, but an attractive model would also be that Ins(1,6)P_2 and Ins(1,5,6)P_3 represent intermediates of a phosphoinositide-independent formation of Ins(1,3,4,5,6)P_5 via L-Ins(1,4,5,6)P_4. Finally, it cannot be excluded that Ins(1,6)P_2 and Ins(1,5,6)P_3 are part of an entirely new branch of inositol phosphate metabolism. We hope that future substrate–product analyses employing isolated inositol phosphate isomers and crude enzyme extracts from avian erythrocytes will elucidate the precise metabolic role of Ins(1,5,6)P_3.

The importance of Ins(2,4,5)P_3 in the metabolism of inositol phosphate remains unclear. For this isomer, which we identified in relatively high amounts in turkey blood, no significant quantities of corresponding dephosphorylation or phosphorylation products, e.g. Ins(2,4)P_2 or Ins(1,2,5,6)P_4, have been found. Ins(2,4,5)P_3 was also identified in fresh blood extracts which were not subjected to several extraction steps (see Table 2, column A). It is therefore unlikely that an extremely large amount of Ins(2,4,5)P_3 is formed from Ins(1,4,5)P_3 or Ins(1,5,6)P_3 by phosphate migration (cf. the Results section). A potential source for Ins(2,4,5)P_3 could be Ins-(1:2cyc,4,5)P_3. Under acidic conditions the cleavage of the cyclic diester bond could result in the formation of some Ins(2,4,5)P_3 in the addition to Ins(1,4,5)P_3 (Hawkins *et al.*, 1987). Downes *et al.* (1988) investigated the hydrolysis of [^{3}H]inositol-labelled D-phosphatidylinositol 4,5-bisphosphate by phosphoinositidase C, employing isolated membranes from turkey erythrocytes. They recorded the formation of only minimal quantities of a compound with the chromatographic and chemical properties of Ins(1:2cyc,4,5)P_3. Even if we assume that, *in vivo*, high enough amounts of this cyclic isomer could accumulate because of slow metabolism (Connolly *et al.*, 1986), we do not know why, under our extraction conditions, so much Ins(2,4,5)P_3 is formed from cyclic InsP_3. As yet the available data give no satisfactory explanation for the high concentration of Ins(2,4,5)P_3 found in avian blood.

The concentrations of inositol phosphate isomers shown in Table 2 might reflect the relative importances of individual metabolic ways of inositol phosphates and their branchings as well as apparent K_m values of enzymes involved. But it should be borne in mind that total concentrations of isomers measured might differ quite markedly from their free concentrations in the cell. The high-affinity binding of InsP_5 to haemoglobin is well known (Isaacks *et al.*, 1977), and our preliminary results (T. Radenberg & G. W. Mayr, unpublished work) have shown that Ins(1,4,5,6)P_4 is also tightly bound to this protein and thus is protected from attack by kinase and phosphatase activities. For other inositol phosphate isomers, binding proteins could exist as well, both in avian erythrocytes and in other cells. The quantitative spectrum of inositol phosphates in avian erythrocytes evaluated here can give a working basis for future studies on the metabolic pathways of inositol phosphates.

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