Confirmation of the identities of inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate by the use of one-dimensional and two-dimensional n.m.r. spectroscopy

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Multinuclear n.m.r. spectroscopy, including the use of two-dimensional methodology, was used to confirm the identity of inositol 1,3,4-trisphosphate and its metabolic precursor inositol 1,3,4,5-tetrakisphosphate. The cyclohexane ring in each molecule exhibits a chair conformation with all phosphate groups occupying equatorial positions.

INTRODUCTION

Since inositol 1,4,5-trisphosphate, $\text{Ins}(1,4,5)\text{P}_3$, has been shown to play an important part as a cellular second messenger (Berridge & Irvine, 1984), much attention has been focused on inositide metabolism, and other inositol phosphates have been isolated and their biochemistry has been studied. Spectroscopic evidence has been presented for the identity of $\text{Ins}(1,4,5)\text{P}_3$ (Lindon *et al.*, 1986), and its stereospecific synthesis has been published (Ozaki *et al.*, 1986). The extraction and separation of two more such phosphates in stimulated tissues have been described (Irvine *et al.*, 1984, 1985, 1986a; Batty *et al.*, 1985), but their identities were elucidated by indirect means, and the proposed structures can only therefore be regarded as preliminary. We have, through the use of one-dimensional and two-dimensional n.m.r. spectroscopy, now confirmed the identities of $\text{Ins}(1,3,4)\text{P}_3$ (I) and its metabolic precursor $\text{Ins}(1,3,4,5)\text{P}_4$ (II) (Fig. 1). The structure of the latter is particularly important in view of its recent identification as the second messenger controlling Ca^{2+} homeostasis at the plasma membrane (Irvine & Moor, 1986).

We should note that n.m.r. spectroscopy, which identifies the chemical environment of magnetic nuclei, is unable to distinguish molecules that are simply mirror images, and hence *D-myo-Ins*(1,3,4) P_3 [which is the same

molecule as *L-myo-Ins*(1,3,6) P_3] has an n.m.r. profile identical with that of *L-myo-Ins*(1,3,4) P_3 [= *D-myo-Ins*(1,3,6) P_3]. No effort has been made to determine the absolute configuration of the inositol phosphates, but the *D* configuration had earlier been assumed at least for $\text{Ins}(1,4,5)\text{P}_3$, reflecting the configuration of the parent phosphoinositide (Grado & Ballou, 1961; Parthasarathy & Eisenberg, 1986), and as $\text{Ins}(1,3,4,5)\text{P}_4$ is prepared by phosphorylation of *D-myo-Ins*(1,4,5) P_3 (see below) then we can assume that the *D* configuration holds for both molecules under study here.

EXPERIMENTAL

Samples (1 μmol ; approx. 400 μg) of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, prepared by phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ with a rat brain supernatant and then when necessary dephosphorylation of the resulting $\text{Ins}(1,3,4,5)\text{P}_4$ by human erythrocyte membranes as described by Irvine *et al.* (1986b), were each dissolved in $^2\text{H}_2\text{O}$ to a concentration of about 2 mM in a 4 mm n.m.r. tube. They were then inserted in a 5 mm n.m.r. tube and used without further treatment other than solution 'pH' adjustment with NaO^2H or ^2HCl . N.m.r. spectra were measured by using a Bruker AM-360 spectrometer at 360.13 MHz for ^1H , at 145.78 MHz for ^{31}P and at 90.57 MHz for ^{13}C . Two-dimensional ^1H - ^1H coupling-constant correlation spectra were taken by using the COSY technique (Bax & Freeman, 1981) and ^{31}P - ^1H two-dimensional chemical-shift correlation spectra were obtained by using the cross-polarization method (Bodenhausen & Freeman, 1977). Additional n.m.r. experiments conducted to aid resonance assignments included homonuclear and heteronuclear spin decoupling and ^1H - ^1H nuclear Overhauser difference studies with the use of standard techniques. A separate sample (approx. 8 μmol) of $\text{Ins}(1,3,4,5)\text{P}_4$ was used for the ^{13}C -n.m.r. experiments, but caused complications in the ^1H -n.m.r. spectrum due to the presence of impurities. Further details are given in the Results and discussion section, and appropriate experimental parameters are given in the Figure captions. All spectra were obtained

Fig. 1. Structures of $\text{Ins}(1,3,4)\text{P}_3$ (I) and $\text{Ins}(1,3,4,5)\text{P}_4$

Abbreviations used: InsP_3 and InsP_4 , inositol tri- and tetrakis-phosphates respectively, with isomeric numbering as appropriate.

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at ambient probe temperatures of approx. 25 °C for ^1H and ^{31}P and approx. 30 °C for ^1H -broadband-decoupled ^{13}C n.m.r.

RESULTS AND DISCUSSION

Identity of $\text{Ins}(1,3,4)\text{P}_3$

The resolution-enhanced ^1H -n.m.r. spectrum of this material is shown in Fig. 2(a). Integration shows there to be six hydrogen atoms all with chemical shifts consistent with the structure. The ^{31}P spectrum is shown in Fig. 2(b), confirming three phosphate groups. The doublet splittings are caused by ^{31}P - ^1H coupling, this interpretation being confirmed by ^1H broadband decoupling. Impurity peaks are observed, and these are ascribed to the $\text{Ins}(1,4,5)\text{P}_3$ isomer (see below). Detailed analysis of the ^1H -n.m.r. spectrum serves to assign all the bands and confirm the positions of phosphorylation. Thus at lowest field, at a chemical shift of δ 4.50, comes H2 showing small spin couplings to H1 and H3 both typical of axial-equatorial interactions. Next, at δ 4.21 is H4 showing three couplings, two axial-axial interactions

Fig. 2. N.m.r. spectra of $\text{Ins}(1,3,4)\text{P}_3$

(a) ^1H -n.m.r. spectrum: 1010 scans, spectrum width 5000 Hz, 32 K data points. Lorentzian-Gaussian resolution enhanced, pH approx. 10. (b) ^{31}P -n.m.r. spectrum: 4829 scans, spectrum width 12 500 Hz, 16 K data points, zero-filled to 64 K, 0.5 Hz line-broadening, pH approx. 10.

Fig. 3. Two-dimensional ^1H - ^1H COSY contour map of $\text{Ins}(1,3,4)\text{P}_3$

There were 200 scans for each of 128 experiments each with 512 data points, spectrum width 518 Hz, zero-filled to 256 points in the F1 domain with sinebell apodization in both domains, pH approx. 10.

with H3 and H5 and a coupling to the phosphate phosphorus atom at C4. Both H5 and H6 are well resolved at higher field, resonating at δ 3.58 and δ 3.86 respectively. The complex multiplet centred near δ 4.0 comprises both H1 and H3, and it is necessary to analyse this band to prove that the oxygen functions at both C1 and C3 are phosphorylated. This was achieved by spin-decoupling the signal from H2 with the resultant simplification showing that both H1 and H3 still manifested two couplings, one to the vicinal hydrogen atom (H6 and H4 respectively) and the other to the phosphate phosphorus atom at C1 and C3 respectively. In addition, because of the pH-dependence of the n.m.r. spectrum, we were able to show that both H1 and H3 are well resolved at about pH 5 and that both are clearly triplets of doublets, as would be expected. The assignments of the proton signals were confirmed by using a two-dimensional COSY experiment, with the result giving the connectivities shown in Fig. 3. All the data are consistent with only one arrangement of nuclei, namely as in $\text{Ins}(1,3,4)\text{P}_3$ or its enantiomer $\text{Ins}(1,3,6)\text{P}_3$. The n.m.r. parameters are collected together in Table 1. Impurity peaks are evident in both the ^{31}P -n.m.r. and the ^1H -n.m.r. spectra, and from the three resolved chemical shifts in the ^{31}P -n.m.r. spectrum at about pH 9 and the shifts and coupling patterns where they can be resolved in the proton spectrum it is most likely that this impurity is the isomeric $\text{Ins}(1,4,5)\text{P}_3$. The bulk sample of $\text{Ins}(1,3,4)\text{P}_3$ for these experiments was not purified by h.p.l.c., and some $\text{Ins}(1,4,5)\text{P}_3$ may have been produced by the human erythrocyte membranes during the hydrolysis of $\text{Ins}(1,3,4,5)\text{P}_4$ (see Irvine *et al.*, 1986b). A further small impurity caused additional complications in the two-dimensional COSY experiment, and this was traced to a resonance present in the $^2\text{H}_2\text{O}$ solvent at about

Table 1. N.m.r. parameters for *Ins(1,3,4)P₃* and *Ins(1,3,4,5)P₄*

Chemical shifts are labelled according to the nucleus being observed and the position number in the *myo*-inositol ring. ¹³C shifts are in p.p.m. quoted relative to tetramethylsilane but referenced to external dioxan at 67.4 p.p.m. ³¹P shifts are in p.p.m. quoted relative to external H₃PO₄. ¹H shifts (δ) are quoted relative to tetramethylsilane but referenced to ²H₂O at δ 4.8. Coupling constants are in Hz, by first-order analysis. Abbreviation: N.R., not resolved.

	<i>Ins(1,3,4)P₃</i>	<i>Ins(1,3,4,5)P₄</i> *
Chemical shifts (p.p.m.)		
H1	4.04†	≈ 4.04
H2	4.50	4.66
H3	4.05†	≈ 4.04
H4	4.21	4.39
H5	3.58	3.96
H6	3.86	≈ 4
P1	4.75	4.27
P3	3.85	4.20
P4	5.35	4.54
P5	—	5.30
Coupling constants (Hz)		
H1–H2	2.8	2.6
H2–H3	2.8	2.6
H3–H4	9.3	9.4
H4–H5	9.1	9.4
H5–H6	9.4	N.R.
H6–H1	9.6	N.R.
H1–P1	8.4	7.4
H3–P3	7.9	7.6
H4–P4	7.4	9.7
H5–P5	—	6.4

* ¹³C chemical shifts for *Ins(1,3,4,5)P₄*: 78.4 (C5), 75.1, 74.5 (C3), 74.1, 73.5 and 71.2 (C2) p.p.m.
† Assignments may be reversed.

δ 2.5 being ‘aliased’ into the spectral region. In addition, the ¹H-n.m.r. spectrum has been measured over the pH range approx. 3.5–10, and the lack of variation in the coupling constants observed confirms that, like *Ins(1,4,5)P₃*, *Ins(1,3,4)P₃* also takes up a conformation with all three phosphate groups equatorial over this entire range.

Identity of *Ins(1,3,4,5)P₄*

The methods used to identify the configuration and conformation of this inositol phosphate follow those used above. The ¹³C-n.m.r. spectrum indicates six non-equivalent carbon atoms all with chemical shifts typical of –CHO– groups, and some showing splittings due to coupling to ³¹P (Fig. 4a). The ³¹P-n.m.r. spectrum shows, as expected, four signals corresponding to the four phosphate groups, all showing coupling to ¹H (Fig. 4b), this again being confirmed by broadband ¹H decoupling (Fig. 4c). Unfortunately, the ¹H-n.m.r. spectrum demonstrates a great deal of overlap, with only two hydrogen atoms being well resolved, although some features can be analysed on the edge of the four-proton band at highest field (Fig. 4d). The presence of four non-equivalent phosphate groups having been shown, this rules out the symmetrical *Ins(1,3,4,6)P₄* and leaves *Ins(1,3,4,5)P₄* together with its mirror image *Ins(1,3,5,6)P₄* and *Ins(1,4,5,6)P₄* and its mirror image

Fig. 4. N.m.r. spectra of *Ins(1,3,4,5)P₄*

(a) ¹³C-n.m.r. spectrum, 111313 scans, spectrum width 5050 Hz, 16K data points, FT size 16K, line-broadening 1 Hz, broadband ¹H decoupling, pH approx. 10. (b) ³¹P-n.m.r. spectrum, spectrum width 12500 Hz, 16K data points, zero-filled to 64K, 1 Hz line-broadening, pH approx. 10. (c) ³¹P-n.m.r. spectrum, ¹H-broadband-decoupled, 140 scans, other parameters as for (b). (d) ¹H-n.m.r. spectrum, 11630 scans, 4K data points, zero-filled to 16K, spectrum width 1080 Hz, Lorentzian-Gaussian resolution enhanced, pH approx. 10.

Fig. 5. Two-dimensional ^1H - ^1H COSY contour map of $\text{Ins}(1,3,4,5)\text{P}_4$

There were 200 scans for each of 128 experiments with 512 data points, spectrum width 371 Hz, zero-filled to 256 points in the F1 domain, sinebell apodization in both domains, pH approx. 10.

$\text{Ins}(3,4,5,6)\text{P}_4$. From this inositol phosphate was prepared the $\text{Ins}(1,3,4)\text{P}_3$ or $\text{Ins}(1,3,6)\text{P}_3$ identified above (Irvine *et al.*, 1986b) and, assuming that there is no phosphate scrambling on dephosphorylation (Batty *et al.*, 1985), it follows that the only possible structure is $\text{Ins}(1,3,4,5)\text{P}_4$. Additional evidence for this assignment comes from a comparison of the ^1H chemical shifts with those in $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ (Lindon *et al.*, 1986). From these data, phosphorylation appears to cause a deshielding of approx. 0.3–0.4 p.p.m. at the proton attached to the same carbon atom as the phosphate group. Using this substituent effect and the observed chemical shifts in the inositol trisphosphates above, one would predict a chemical shift for H3 of about δ 4.0 in $\text{Ins}(1,3,4,5)\text{P}_4$, close to that observed, whereas in $\text{Ins}(1,4,5,6)\text{P}_4$ H3 would resonate at about δ 3.7, where no signals appear. These chemical-shift arguments, together with analysis of coupling patterns, aid the assignment of the ^1H -n.m.r. spectrum. The signal at δ 4.66 arises from H2, confirming it to be in an equatorial position from the H2–H3 and H2–H1 coupling constants. The band at δ 4.39 comes from H4, the low-field part of the four-proton multiplet near δ 4.0–4.1 being due to H1 and H3 and the highest-field part arising from H5. These assignments are confirmed by the homonuclear spin-decoupling experiments and the two-dimensional COSY spectrum (Fig. 5). Having assigned the ^1H -n.m.r. spectrum, the assignments of the ^{31}P signals follow from the ^{31}P - ^1H correlation experiment (Fig. 6). In addition, the ^{13}C -n.m.r. spectrum (Fig. 4a) was measured in an attempt to obtain $^{13}\text{C}/^{31}\text{P}$ coupling constants to provide detailed conformational information, and some peaks can be assigned through a two-dimensional correlation study (results not shown). All the n.m.r. parameters are summarized in Table 1.

Fig. 6. Two-dimensional ^1H - ^{31}P chemical-shift correlation contour map for $\text{Ins}(1,3,4,5)\text{P}_4$ showing connectivities via $^3J_{\text{H-P}}$

There were 128 scans for each of 128 experiments with 1000 data points, spectrum width 304 Hz, zero-filled to 256 points in the F1 domain, sinebell apodization in both domains.

After the completion of this work we have learnt of independent experiments by Cerdan *et al.* (1986) in which they measure and interpret the n.m.r. spectra of a number of inositol phosphates, including $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5)\text{P}_3$. Their assignments for $\text{Ins}(1,3,4,5)\text{P}_4$ taken at pH 8.0 agree with those quoted above, allowing for differences due to pH effects. This is most noticeable for ^{31}P chemical shifts, where ours show more deshielding and are in a different order from theirs. The two sets of data are not inconsistent if one takes into account the fact that such shifts move to lower field on increasing the pH, as exemplified by inositol hexakisphosphate (Emsley & Niazi, 1981). In addition, the order of the shifts is markedly affected by the pH, because the various interacting phosphate groups are likely to have quite different $\text{p}K_a$ values, again by comparison with InsP_6 (Isbrandt & Oertel, 1980; Emsley & Niazi, 1981). All studies to date on inositol phosphates that have involved the measurement of ^{31}P -n.m.r. parameters demonstrate the lack of diagnostic use of both ^{31}P shifts and ^{31}P - ^1H coupling constants because of their significant pH-dependence. The effect of pH may account for the very low value of $^3J_{\text{P-H}}$ that we measured for one phosphate group in $\text{Ins}(1,4,5)\text{P}_3$ (Lindon *et al.*, 1986). The effect of phosphorylation on ^1H chemical shifts in this series of molecules, estimated as 0.3–0.4 p.p.m. at the nearest proton, is also supported by the more detailed parameters given by Cerdan *et al.* (1986).

In conclusion, these results confirm the preliminary structural determinations of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine *et al.*, 1984; Batty *et al.*, 1985). In conjunction with our earlier work on $\text{Ins}(1,4,5)\text{P}_3$ (Lindon *et al.*, 1986), they show that the $\text{InsP}_3/\text{InsP}_4$ pathway proposed by Irvine *et al.* (1986a), whose function is to generate $\text{Ins}(1,3,4,5)\text{P}_4$, an intracellular

second messenger in its own right (Irvine & Moor, 1986), is structurally correct.

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