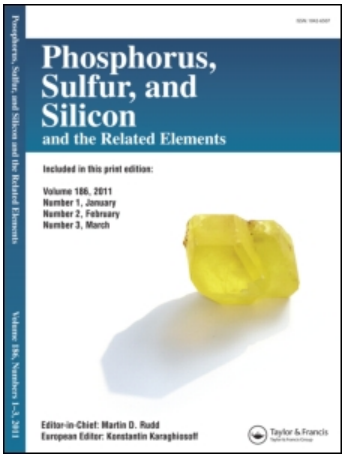

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THE STRUCTURE OF *myo*-INOSITOL HEXAPHOSPHATE IN SOLUTION: ^{31}P N.M.R. INVESTIGATION

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THE STRUCTURE OF *myo*-INOSITOL HEXAPHOSPHATE IN SOLUTION: ^{31}P N.M.R. INVESTIGATION

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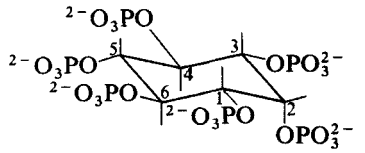
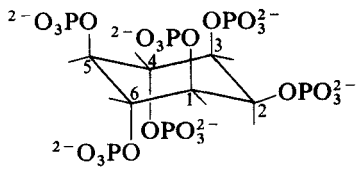
Changes in the ^{31}P n.m.r. spectrum of *myo*-inositol hexaphosphate at different pH reveal that the conformation of this molecule varies with its degree of protonation. Above pH 12 it has the equatorial structure (Ie), below it has the axial conformation (Ia) which it retains to pH 5 when it reverts to Ie on the addition of a seventh proton. These changes are explained by strong hydrogen bonding between phosphate groups. There is also evidence that at low pH (2) the molecule again reverts to Ia.

A recent book is devoted entirely to the inositol phosphates.[1] There are nine inositols,[2] of general formula $C_6H_6(OH)_6$, and for some of them the mono-, di-, tri-, tetra-, penta-, and hexa-phosphates are known, including positional and optical isomers. From the multiplicity of possible compounds one stands alone as being biologically the most important and that is *myo*-inositol hexaphosphate, *myo*-IP_6. Known for over a century,[3] this material is produced by plants primarily as a phosphate store, although it has several physiological roles.[1,2] In animals it has a beneficial role as a hemoglobin cofactor and a possible detrimental role as a scavenger of certain metals such as Ca, Zn, Mn, Fe, and Cu with whose ions it can form very insoluble salts.[4] It is also a major reservoir of phosphate in soils, along with other inositol phosphates from microbial sources, and by virtue of its slow rate of hydrolysis and its insolubility as Ca, Fe or Al salts, it can become one of the major phosphate components of fertile soil.[5]

myo-Inositol hexaphosphate can have one of two conformations, Ia, in which five phosphate groups are axially orientated and one is equatorial, or Ie, in which five are equatorial and one is axial. Theory would suggest Ie as the preferred orientation whereby steric crowding of the phosphate groups is minimized. *myo*-Inositol itself has this configuration.[6]



Ie



Ia

On the basis of acid strengths it was originally deduced that structure Ia was the more likely for *myo*-IP_6, there being transanular hydrogen bonding between the phosphate groups on C1 and C3 and C4 and C6.[7] This conformer was supported by the x-ray crystal structure of the salt *myo*-$IP_6Na_{12} \cdot 38H_2O$,[8] which still showed Ia despite there being no direct hydrogen bonding across the ring as envisaged by Barre *et al.*[7] That *myo*-IP_6^{12-} should prefer Ia was explained as due to a lessening of repulsion between the doubly charged phosphate groups even though this produced some 1,3-syn-axial strain between oxygen atoms bonded to the ring. This strain however, was partly counterbalanced by these oxygen atoms being incoporated

into the co-ordination sphere of a sodium atom. Because of the environment of 38 water molecules surrounding the inositol hexaphosphate ion it was reasoned that the same conformation would be the preferred one in aqueous solution at high pH.[8]

This suggestion was questioned by Costello *et al.*, who observed the ^{31}P n.m.r. spectrum of *myo*-IP_6 at different pHs from 1 to 12.[9] They concluded that conformer Ie was the preferred one over the whole pH range. This evidence need not, of course, be in conflict with the solid state structure. Lattice energy could supply the difference between Ie and Ia to enable the latter to pack more effectively. However, it seems possible that other factors such as hydrogen bonding might intervene in the Ie $\leftrightharpoons$ Ia equilibrium even in solution. We therefore reinvestigated the ^{31}P n.m.r. spectrum of *myo*-IP_6 over the pH range from 13–1.2 concentrating mainly on the high pH region.

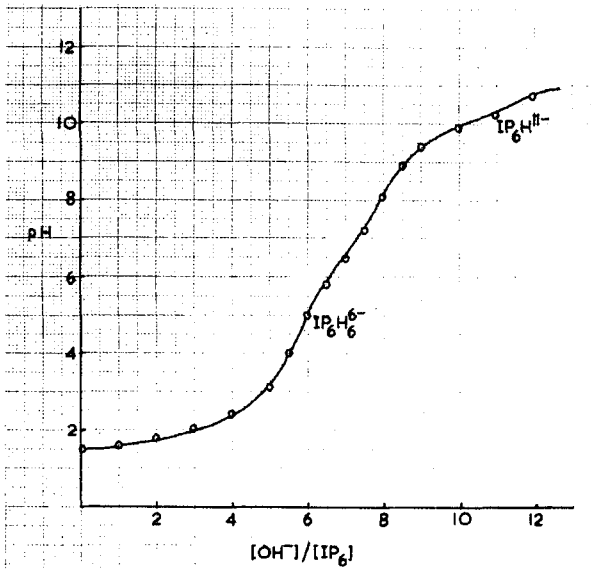


Figure 2 Titration curve of IP_6H_{12} vs. $[Bu_4N]OH$.

EXPERIMENTAL

Materials

Sodium inositol hexaphosphate, $C_6H_6(OPO_3Na_2)_6 \cdot n\text{-}H_2O$, was purchased from BDH Biochemicals. Careful drying and treatment with $SOCl_2$ to remove residual water showed n to be 15. A 0.1 M solution of the sodium salt was converted to the acid by ion exchange (Amberlite resin IR-120(H)) and the pH of the solution was adjusted by titration with 1.74 M tetra-*n*-butylammonium hydroxide solution.

Spectra

^{31}P n.m.r. spectra were recorded on a Bruker HFX90 spectrometer at 307°K from 0.10 M solutions containing 50% D_2O. Peak positions were measured in p.p.m. from 85% H_3PO_4 as external standard: filter bandwidth 7500 Hz/cm; sweepwidth 6024 Hz/cm; offset 4500 Hz; no systematic noise reduction. Pulse-Fourier transform: 12.5 μsec pulsewidths, 30° flip angle; 8 K points; time constant −2 sec; 256 or 512 scans per spectrum; broad band decoupled. Figure 1 shows the type of spectra obtained at various pHs. Table II lists the chemical shifts, etc.

pH was measured on a Pye pH meter.

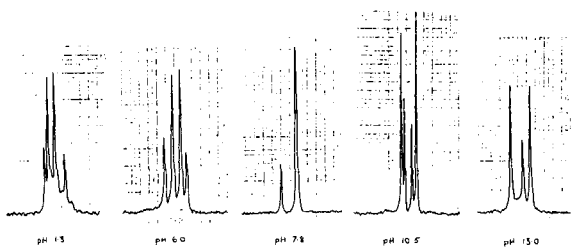


FIGURE 1 ^{31}P n.m.r. spectra of $[IP_6H_i]^{i-12}$ at different pH values.

RESULTS AND DISCUSSION

The shape of the titration curve, Figure 2, is identical with those reported by Barre *et al.*[7] many years ago. However, we were in the position of knowing exactly the concentrations of *myo*-IP_6 solutions whereas the earlier work showed different results according to the source of the material. Nevertheless they were able to deduce that six protons in *myo*-IP_6H_{12} were very acidic with pK_a's 1.84 and titrateable in the range 1.8–4.9; two protons were weakly acidic, pK_a's 6.3, titrateable in the range 4.9–8.0; and that four protons were very weakly acidic, pK_a's 9.7, titrateable in the range 8.0–11.5.

On the other hand Costello *et al.*[9] reported pK_a's of 1.1, 1.5 (2H), 1.7, 2.1 (2H), 5.7, 6.85, 7.60, 10 (2H) and 12.0 which can be grouped as six strongly acid, three weak and three very weak. Their results were obtained from inflection points in the pH vs $\delta(^{31}P)$ curves for the various phosphates. Our direct titration curve agrees with Barre's results. This, combined with the pH vs. $\delta(^{31}P)$ profiles of Figure 3 allow us to deduce the order of removal of protons from *myo*-IP_6H_{12} is as shown in Table I. It is somewhat surprising to find that P5, i.e. the phosphate group on C5, appears to have *three* hydrogens. The explanation for this is that proton migration within the ion

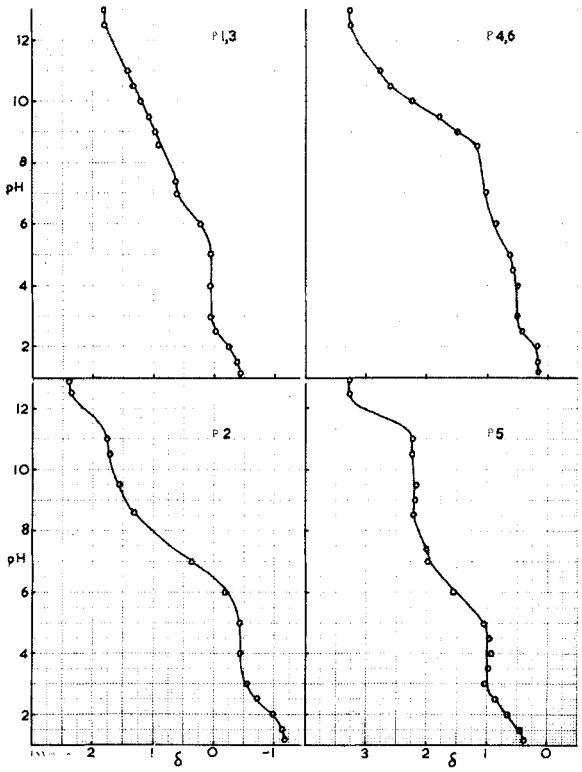
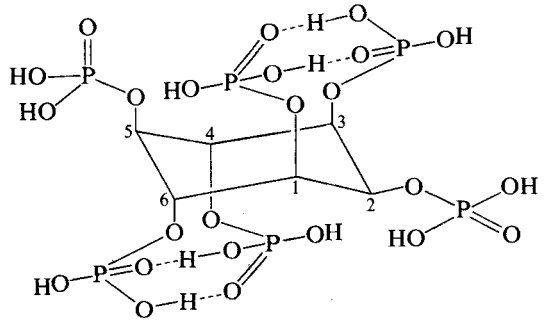


FIGURE 3 pH vs. (^{31}P) profiles of individual phosphate centres in $[IP_6H_i]^{i-12}$.

occurs at *ca* pH 8. The information in Table I is arranged in order of proton addition to *myo*-IP_6^{12-}.

Barre *et al.*[7] explained their observations in terms of structure Ia with double hydrogen bonds between P1 and P3, and P4 and P6, involving the four protons which are only very weakly acidic: (II).



(II)

The numerical values of the chemical shifts and coupling constants of the phosphates over the pH range 1–13 are listed in Table II and displayed in Figure 4. The coupling constants J_{POCH} in the

TABLE I

Sequence of addition of protons to *myo*-IP_6^{12-}

$[myo\text{-}IP_6H_i]^{i-12} + H^+ \rightarrow [myo\text{-}IP_6H_{i+1}]^{i-11}$

pH	Ion	Last proton added to phosphate centre
12	IP_6H^{11-}	P5
10.5	$IP_6H_2^{10-}$	P4 or P6
9.5	$IP_6H_3^{9-}$	P1 or P3
8.0	$IP_6H_4^{8-}$	P4 or P6[a]
ca 7	$IP_6H_5^{7-}$	P2
ca [6]	$IP_6H_6^{6-}$	P5
5–3	$IP_6H_7^{5-}$	? (P5)[b]
2.5	$IP_6H_8^{4-}$	P2
2.5–2	$IP_6H_{10}^{2-}$	P4 and P6
2–1.5	IP_6H_{12}	P1 and P3

[a] Proton on P5 migrates to P1 or P3 at this pH: see text.

[b] Seventh proton settles on P5 only when eighth proton enters molecule.

pH range 2–6 could not be recorded due to overlap. The values and assignments do not agree with those of Costello *et al.*[7] nor do we interpret them as they did solely in terms of Ie.

Factors Influencing δ(^{31}P) of the Phosphate Group

These are (i) those arising from the molecular environment of which the degree of protonation is more important than the conformation of the phosphate with respect to the ring; and (ii) those arising from the solvent environment of which the counter cation may be more important than the solvent itself especially if the cation is an ion like Na^+.

The importance of the degree of protonation was demonstrated for orthophosphate itself where removal of the first proton from H_3PO_3 caused a high frequency shift of 0.5 p.p.m. while removal of the second and third protons both caused further shifts each of 2.5 p.p.m.[10] The effect of counter cation has been demonstrated by Glonek *et al.* who found that substituted ammonium ions were best, the ion having least effect being Bu_4N^+.[11]

Hydrogen Bonding Involving Phosphate Groups

It is possible to envisage various kinds of hydrogen bonding between two phosphate groups (IV–VI). Fully protonated (IV) the two groups can form two

TABLE II

^{31}P n.m.r. data

pH	Ion	[δ/ppm 85% H_3PO_3; J_{POCH}/Hz; Integral: location]
13.0	IP_6^{12-}	[3.27; 10.3; 3P; P4, 5, 6] [2.34; 11.8; 1P; P2] [1.74; 10.3; 2P; P1, 3]
12.5		[3.27; 10.3; 3P; P4, 5,6] [2.34; 11.8; 1 P; P2] [1.78; 10.3; 2P; P1,3]
11.0	IP_6H^{11-}	[2.79; 8.8; 2P; P4,6] [2.14; 8.8; 1P; P5] [1.77; *ca* 15; 1P; P2][1.41; 8.8; 2P; P1,3]
10.5	$IP_6H_2^{10-}$	[2.58; 8.8; 2P; P4,6] [2.14; 10.3; 1P; P5] [1.70; 13.2; 1P; P2] [1.33; 10.3; 2P; P1,3]
9.5	$IP_6H_3^{9-}$	[2.14; 8.8; 1P; P5] [1.77; 8.8; 2P; P4,6] [1.53; 10.3; 1P; P2] [1.05; 8.8; 2P; P1,3]
9.0		[2.18; 8.8; 1P; P5] [1.45; 8.8; 3P; P2,4,6] [0.97; 8.8; 2P; P1,3]
8.6	$IP_6H_4^{8-}$	[2.22; –; 1P; P5] [1.33; –; 1P: P2] [1.13; –; 2P; P1,3] [0.89; –; 2P; P1,3]
7.8	$IP_6H_5^{7-}$	[2.18; –; 1P; P5] [0.97; –; 5P; P1,2,3,4,6]
7.4		[1.98; –; 1P; P5] [1.01; –; 2P; P4,6] [0.65; –; 3P; P1,2,3]
7.0		[1.98; –; 1P; P5] [1.09; –; 2P; P4,6] [0.61; –; 2P P1,3] [0.36; –; 1P; P2]
6.0	$IP_6H_6^{6-}$	[1.53; 5.9; 1P; P5] [0.80; 7.4; 2P; P4,6] [0.20; 7.3; 2P; P1,3] [–0.20; –; 1P; P2]
5.0		[1.05; –; 1P; P5] [0.52; –; 2P; P4,6] [0.00; –; 2P; P1,3] [–0.48; –; IP; P2]
4.5		[0.97; –; 1P; P5] [0.48; –; 2P; P4,6] [0.00; –; 2P; P1,3] [–0.48; –; 1 P; P2]
4.0		[0.93; –; 1P; P5] [0.48; –; 2P; P4,6] [0.04; –; 2P; P1,3] [–0.44; –; 1P; P2]
3.5		[0.97; –; 1P; P5] [0.44; –; 2P; P4,6] [0.00; –; 2P; P1,3] [–0.52; –; 1P; P2]
3.0	$IP_6H_7^{5-}$	[1.05; –; 1P; P5] [0.52; –; 2P; P4,6] [0.04; –; 2P; P1,3] [–0.52; –; 1P; P2]
2.5	$IP_6H_8^{4-}$	[0.88; –; 1P; P5] [0.40; –; 2P; P4,6] [0.08; –; 2P; P1,3] [–0.73; –; 1P; P2]
2.0	$IP_6H_{10}^{2-}$	[0.68; –; 1P; P5] [0.16; –; 2P; P4,6] [–0.28; –; 2P; P1,3] [–1.01; –; 1P; P2]
1.5		[0.44; –; 1P; P5] [0.16; –; 2P; P4,6] [–0.36; –; 2P; P1,3] [–1.17; –; 1P; P2]
1.2	IP_6H_{12}	[0.36; 8.8; 1P; P5] [0.12; 8.8; 2P; P4,6] [–0.44; 8.8; 2P; P1,3] [–1.21; 8.8; 1P; P2]

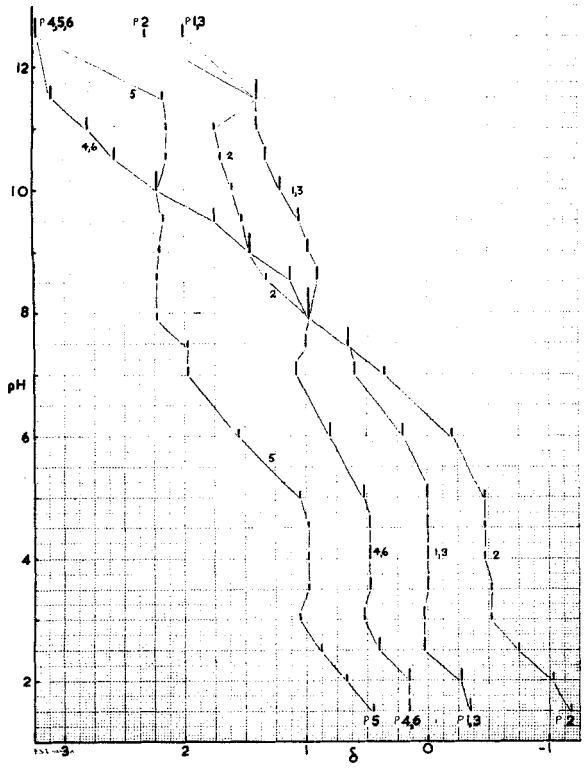
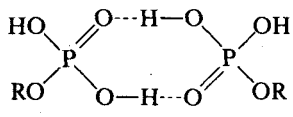
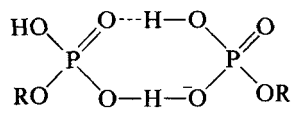


FIGURE 4 The ^{31}P n.m.r. data at different pH values.

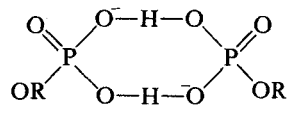
normal hydrogen bonds in a cyclic arrangement similar to the well-known carboxylic acid cyclomers. Such cyclic dimers are reported for $(p\text{-}ClC_6H_4O)_2PO_2H$.[12] Types (V) and (VI) contain



(IV)



(V)

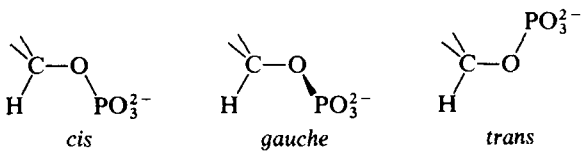


(VI)

one and two very strong hydrogen bonds for which some examples are known.[13,14] It is our belief that strong hydrogen bonds are responsible for holding *myo*-IP_6 in the Ia configuration in solution at pH 12–5.

The Coupling Constant J_{POCH}

It is possible to imagine three orientations of the phosphate centre and the C—H bond of the ring:



The most likely arrangement is *cis* on steric grounds with the *gauche* a possibility, and *trans* highly unlikely especially for any group axially placed. The x-ray crystal structure of *myo*-IP_6Na_{12} showed all *cis* arrangements.[8]

The spectrum of the anion *myo*-IP_6^{12-} show two values for J_{POCH}, 10.3 Hz for five of the phosphate groups and 11.8 Hz for the sixth. In this anion there is no intramolecular hydrogen bonding to deflect the phosphates from this *cis* orientation so that these values represent $J_{POCH\,cis}$ to the two kinds of phosphate group, which are equatorial and axial in conformer Ie (see below).

When there are OH groups in the molecule then intramolecular hydrogen bonds can form and phosphate groups are deflected into a *gauche* position in order to participate. Table II reveals J_{POCH} values of 8.8 Hz at several pH's from 11.0 down to 1.0 when there is full protonation. This value we assign to $J_{POCH\,gauche}$, and take it as a sign of a phosphate involved in hydrogen bonding in conformer Ia.

Other values for the coupling constant, such as 5.9 Hz and 7.4 Hz observed at pH 6 are taken as *gauche*, maybe in conformer Ia or Ie. Between pH 8.5 and 2.5 the signals in the spectrum are wholly or partly overlapping or are broad so that coupling constants are not measurable for all the phosphate groups.

Since we are primarily concerned with the high pH range the data of Table II are interpreted from the top downwards.

myo-IP_6^{12-}: this is the species in solution at pH 12.5 and above. The 3:1:2 pattern of signals enables identification of the phosphates thus: P4, P5 and P6 have identical environments being flanked on either side by phosphates with the same environment; P2, the unique phosphate; and P1 and P3, which have identical environments and are flanked by one phosphate of the same environment and one of the opposite kind. Although the x-ray picture shows *myo*-IP_6^{12-} to be Ia in the solid phase[8] we believe that in solution it prefers Ie. We argue this on the basis of what happens at lower pH values, but in any event the crystal forces which are identified as determining Ia, such as co-ordination to Na^+, no longer apply since the counter cation here is Bu_4N^+.

Thus in strongly alkaline solution *myo*-IP_6^{12-} has three equatorial phosphates on C4, 5, 6 at highest frequency, the single axial phosphate at lower frequency and the remaining two equatorial phosphates at the lowest frequency. Coupling constants support this identification. The general concept of axial phosphates being more deshielded than equatorial ones does not seem to be universally valid.[1,5,9]

myo-IP_6H^{11-}: with the addition of a proton to the ion *all* the signals in the n.m.r. spectrum move to lower frequencies, by *ca.* 0.4–0.6 p.p.m., except the phosphate to which the proton is bound, P5, which shifts by 1.13 p.p.m. and moves clear from the P4, P6 signal. Because all the phosphates are affected by the entry of this single proton we take it as indicative of a change in conformation from Ie to Ia. The addition of subsequent hydrogen only markedly affects the phosphate to which it is attached.

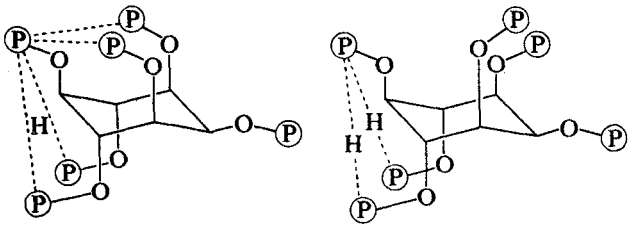
The presence of a proton on the P5 phosphate decreases the J_{POCH} values of all phosphates to 8.8 Hz except that on P2, the only equatorial group, which actually increases. The inference is that the proton is hydrogen bonding between P5 phosphate and the other phosphates of P4, P6, P1 and P3 something which it can only do if all these groups are *axial* as in Ia, see VII. If they were equatorial as Ie requires then hydrogen bonding could only occur between P5 and the adjacent phosphates of P4 and P6.

If our reasoning for *myo*-IP_6H^{11-} is correct, i.e. that it has structure Ia with both adjacent and transanular strong hydrogen bonding between axially placed phosphates, then the structure of *myo*-IP_6^{12-} must be Ie. Previous investigators of the ^{31}P n.m.r. spectra concluded that the structure was Ie irrespective of any degree protonation.[9]

myo-$IP_6H_2^{10-}$: on the addition of a second proton at pH 10.5 there is a marked upfield shift in the signals of P4 and P6 phosphates. At lower pH values their combined signal eventually moves to the low frequency side of that P5. However, the most interesting feature of the spectrum is the

coupling constant values of P5, P1 and P3 phosphates which return to 10.3 Hz indicating $J_{POCH\,cis}$ while only those of P4 and P6 remain at 8.8 Hz indicating $J_{POCH\,gauche}$. (The value for the P2 phosphate is difficult to measure due to overlap but it is *ca.* 15 Hz.) These J values show hydrogen bonding between P5 and both P4 and P6 phosphates which is symmetrical with respect to P5. This explains why this shows $J_{POCH\,cis}$, see VIII.

An alternative suggestion of a transanular double hydrogen bridge between P4 and P6 would also explain the coupling constants. However, this would require proton migration from P5 and presumably a downfield shift for this phosphate which is not observed.

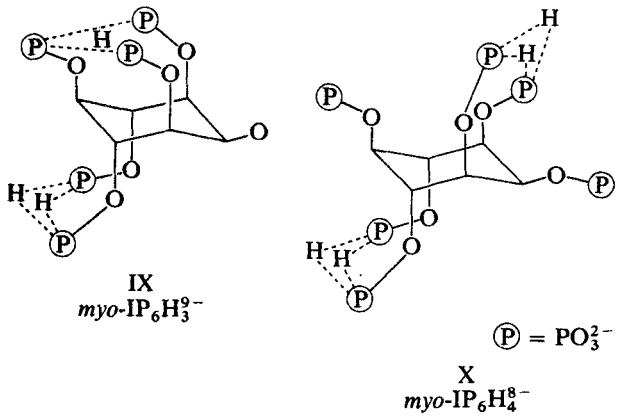


VII
myo-IP_6H^{11-}

VIII
myo-$IP_6H_2^{10-}$

Ⓟ = PO_3^{2-}

myo-$IP_6H_2^{9-}$: addition of a third proton at pH 9.5 causes J_{POCH} of P5, 1 and 3 to revert to 8.8 Hz leaving only P2 at 10.3 Hz. Again strong hydrogen bonding is involving P1 and P3. The continued upfield shift of the P4, 6 signal shows that the third hydrogen is attached here so that these can now form a double bridge of hydrogen bonding structure VI with P5, 1 and 3 sharing a proton as in IX.



IX
myo-$IP_6H_3^{9-}$

Ⓟ = PO_3^{2-}

X
myo-$IP_6H_4^{8-}$

myo-$IP_6H_4^{8-}$: the fourth hydrogen, entering the molecule at pH 8, produces a spectrum in which the signals for the phosphates of P1, 2, 3, 4 and 6 are merged. The P5 remains separate and is slightly shifted *downfield*. It seems most likely that there are now two double strong hydrogen bond bridges of type VI between P4 and P6 and P1 and P3 (X), giving rise to coincident signals that also overlap the P2 signal which is in this region of the spectrum. The *downfield* shift of P5 can be explained by proton migration from it to complete the P1–P3 bridge. This also explains why this phosphate centre appears tribasic with three pH regions showing marked $\delta(^{31}P)$ changes at ca. 12, 6.5 and 2.2.

At pH 7.5 the signal of integral ratio 5 splits into a 2 and 3 integral pair of peaks and at 7.0 the latter separates into an upfield singlet (P2) and one for P1, 3. This relative positioning of the peaks of P5; P4, 6; P1, 3; P2 as a 1:2:2:1 pattern now remains unchanged down to pH 1.0.

At pH 7.0 the anion is *myo*-$IP_6H_5^{7-}$ and the upfield shift shows that the fifth proton is attached to the P2 phosphate. Here it can only engage in strong hydrogen bonding with P1 and P3. Similarly, when at pH 6, the sixth proton is attached to P5 it must disrupt the P4–P6 double hydrogen bond bridge if it is to engage in strong hydrogen bonding. As observed there is an upfield shift of all the signals as the sixth proton enters and the coupling constants drop to 5.9 (P5) and 7.4 Hz (P1, 3, 4, 6). That of P2 could not be measured.

With *myo*-$IP_6H_6^{6-}$ each phosphate can act as a hydrogen bond donor and acceptor and the hydrogen bonding is at a maximum of 12 strong hydrogen bonds. On the n.m.r. timescale these are short lived and what the spectrum reveals is the 1:2:2:1 pattern predicted for Ia in which the hydrogen bonding is averaged out.

As Table II shows there is no change in δ values over the pH range 5.0–3.0 except that the signals become broad. That there is no upfield shift on addition of the seventh proton suggests a change in conformation of the molecule from Ia to Ie, the downfield effect of which counter-balancing the upfield shift of this seventh proton. Moreover since no particular phosphate seems to be affected we can infer that the hydrogen bonding is delocalised around the periphery of the molecule as Ie would require. This broadens the signals.

At pH 2–5 we have *myo*-$IP_6H_8^{4-}$ and $\Delta\delta$ shows that the eighth proton settles on P2 phosphate and this causes the previous proton to localise on P5. The remaining four protons add to P4 and P6, then P1 and P3. These additions cause the smallest

upfield shifts. The signals become sharper and the coupling constants are all 8.8 Hz indicative of $J_{POCH\ gauche}$ in a molecule of conformation Ia again. The upfield shift is not pronounced so that we cannot be certain that there is a final change to Ia for the acid *myo*-IP_6H_{12}.

A separate piece of evidence to support a change of conformation to Ie between pH 5 and *ca* 2 is the change in the rate of hydrolysis which occurs over this range. Since an axially placed group is more susceptible to attack than an equatorially placed one then Ia should hydrolyse more readily than Ie. If so then at very high pH and between pH 5–2 the rate of hydrolysis should be less than that at other pHs. Although the data are sparse it has been reported that the extent of hydrolysis of *myo*-inositol hexaphosphate is zero at pH 12, increases at lower pH but then *decreases* below pH 4 down to pH 1 when it rises again.[16] While this does not exactly reflect our results it does fit well without our contention of a Ie structure above 12 and between 5 and 2. Too much cannot be read into these results since there are other factors such as charge to be taken into account and the hydrolysis was performed at 100° which may affect the Ia $\rightleftharpoons$ Ie equilibrium.

The concept of changes in ring conformation with changes in pH is supported by work on the optical rotation of the potassium salt of L-chiroinositol hexaphosphate. This has variation in the optical rotation with pH that has led to the suggestion that the conformer is 4 equatorial-2 axial at low pH and 4 axial-2 equatorial at high pH (the two different groups are on adjacent carbons).[17] This is supported by ^{31}P n.m.r. observations.[18] This behaviour of mainly axial conformation at high pH and the reverse at low pH parallels our results for *myo*-inositol hexaphosphate and we think that again strong hydrogen bonding between axial groups could be responsible.

In a recent paper Isbrandt and Oertel[19] have also reported on the ^{31}P, ^{13}C and Raman spectra of sodium inositol hexaphosphate in aqueous solution over a range of pH and come to the conclusion that the molecule prefers the axial conformation (Ia) at pH above 9·4 and the equatorial (Ie) below this. The system is not strictly comparable with that reported here since the sodium ion is capable of influencing the conformation as it does in the solid, and this they recognize as an important factor. They found no evidence of a relaxation to Ie for the ion $1P_6^{12-}$ at very high pH as we do, but this may not occur in the presence of Na^+. They also find that the P2 phosphate has an apparent basicity of 3 in addition to P5. In this and in other details, such as the assigning of chemical shifts, Isbrandt and Oertel differ from the results reported here.

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