myo-Inositol 1,4,5-Triphosphate and Related Compounds' Protonation Sequence: Potentiometric and ^{31}P NMR Studies

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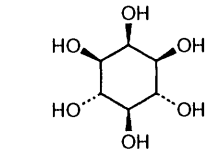
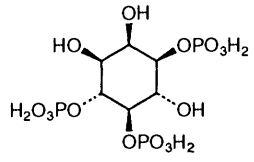
The protonation sequence of *myo*-inositol 1,4,5-triphosphate [Ins(1,4,5)P_3], of its dehydroxylated analogue, Cyhx(1,2,4)P_3, of two diphosphorylated inositol phosphates, Ins(1,4)P_2 and Ins(4,5)P_2 and of one inositol monophosphate, Ins(1)P_1, have been determined. Potentiometric and ^{31}P NMR studies have been performed in a 0.1 mol dm^{-3} solution of tetraethylammonium perchlorate 25 °C (medium 1) and, in addition, for Ins(1,4,5)P_3, in a 0.2 mol dm^{-3} KCl solution at 37 °C (medium 2). In the case of the inositol diphosphate, microconstants related to each individual phosphate could be calculated and interpreted according to the position of the phosphates around the inositol ring. Cyhx(1,2,4)P_3 bears an independent phosphate (P4) and two additional phosphates (P1 and P2) equally sharing the bound protons. For Ins(1,4,5)P_3, strong interactions between the phosphate groups are possible, due to the presence of the hydroxy groups. The chemical shifts of the monoanionic and dianionic phosphate forms of the studied compounds are discussed. The superimposition of binding data with the NMR titration curves of Ins(1,4,5)P_3 emphasizes the particular importance of P5 in binding of Ins(1,4,5)P_3 to its receptor.

The role in cell signalling played by D-*myo*-inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] has been widely demonstrated.[1-10] Ins(1,4,5)P_3 is known to act through mobilization of intracellular calcium stores, after binding to a specific endoplasmic reticulum membrane receptor. Particularly interesting are the facts that (i) calcium itself participates in some cases in a regulation mechanism,[11-13] (ii) potassium ions or other monovalent cations are needed to allow the Ca^{2+} release mediated by Ins(1,4,5)P_3,[12] (iii) the pH highly affects the binding of Ins(1,4,5)P_3 to its receptor.[14-16] Taking into account the structure of this compound, consisting of an inositol ring surrounded by three phosphate groups (see structure), it is likely that the presence of alkali or alkaline-earth cations, as well as the hydrogen ion concentration, greatly influence the nature of the species in the intracellular medium and hence the biological activity of the messenger. Thus, the knowledge of the equilibria taking place in such a medium and the associated stability constants may contribute to a better understanding of the events occurring at a molecular level. Previous studies[17,18] carried out in our laboratory by potentiometric methods have led to protonation and complexation constants of the Ca^{2+}-H^+-Ins(1,4,5)P_3 system. These constants describe macroscopically the interactions between the components of the system, but cannot give any information at a microscopic level. Such information can be provided by NMR spectroscopy, which is a useful technique to determine the protonation sequence of polyprotic acids,[19-23] and allow a more thorough investigation of the complexation reactions.

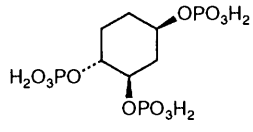
For inositol phosphates as for other organic phosphates, ^{31}P NMR measurements give access to chemical shifts of the phosphorus nuclei which depend mainly on the electronic effects accompanying the deprotonation of the phosphate groups.[20-24] By following the chemical shift of each resonance over a wide pH range, it is possible to obtain for a given pH value a precise description of the ionization state of the inositol phosphate and, in favourable cases, to determine the protonation constants of each individual phosphate moiety. The chemical shift observed for any resonance δ_i^{obs}, is the weighted average of shifts for the appropriate protonated and deprotonated forms,[20,22] and is defined in eqn. (1); where $f_{i,p}$ and



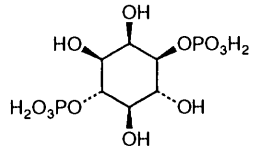
myo-inositol



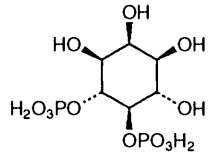
Ins(1, 4, 5)P_3



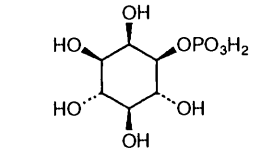
Cyhx(1, 2, 4)P_3



Ins(1, 4)P_2



Ins(4, 5)P_2



Ins(1)P_1

$$\delta_i^{obs} = f_{i,p} \times \delta_{i,p} + f_{i,d} \times \delta_{i,d} \quad (1)$$

$f_{i,d}$ respectively correspond to the protonated and deprotonated fractions of the phosphate in position i, and $\delta_{i,p}$ and $\delta_{i,d}$ are the chemical shifts of the mono protonated and fully deprotonated forms of the phosphate. As the protonation of the phosphates, rather than the deprotonation, is considered, substitution of $f_{i,d}$ by $1 - f_{i,p}$ in eqn. (1), gives eqn. (2). If N is the

$$f_{i,p} = \frac{\delta_i^{obs} - \delta_{i,d}}{\delta_{i,p} - \delta_{i,d}} \quad (2)$$

number of phosphates that the molecule bears, and in the

absence of equivalent groups, the mean number of protons bound per mol of inositol phosphate, $\bar{p}$, can easily be derived from eqn. (3). On the other hand, $\bar{p}$ can be obtained directly

$$\bar{p} = \sum_{i=1}^{i=N} f_{i,p} \quad (3)$$

from potentiometric measurements using eqn. (4);

$$\bar{p} = \frac{C_H - [H^+] + [OH^-]}{C_L} \quad (4)$$

where C_H and C_L correspond to the analytical concentrations of acid and ligand respectively. A good agreement between both representations of $\bar{p}$ *versus* pH as determined using these two different techniques should validate the assumptions made for the NMR calculations, *i.e.* that the chemical shifts are largely dependent on the protonation state of the phosphate groups.

In this work, we report the results of potentiometric determinations followed by ^{31}P NMR titrations, made in the same conditions, on various inositol phosphates and derivatives of biological interest. The compounds under study include two triphosphorylated derivatives, Ins(1,4,5)P_3, and its dehydroxylated analogue, cyclohexane-1,2,4-triol triphosphate [Cyhx-(1,2,4)P_3], two diphosphorylated inositol phosphates, Ins-(1,4)P_2 and Ins(4,5)P_2, and one inositol monophosphate, Ins(1)P_1. In addition to the determination of the protonation sequence of these compounds, the influence of the number and the position of the phosphate groups, as well as the presence of the hydroxy groups around the inositol ring are discussed.

To measure the 'intrinsic' acid–basic properties of the compounds, the studies were performed in 0.1 mol dm^{-3} tetraethylammonium perchlorate (Et_4NClO_4) solution at 25 °C (medium 1). In the case of Ins(1,4,5)P_3, a more physiological medium (0.2 mol dm^{-3} KCl at 37 °C, medium 2) has also been used to explore the influence of alkali cations, and to allow the description of the ionization state of this ligand under near biological conditions.

Determination of Macroscopic and Microscopic Constants.— The overall protonation process can be described by the general equilibrium

$$L^{n-} + yH^+ \rightleftharpoons H_yL^{(n-y)-}$$

which characterizes the constant β_y. n represents the charge of the deprotonated ligand L, and $0 < y < n$. If the stepwise protonation is considered, the equilibrium

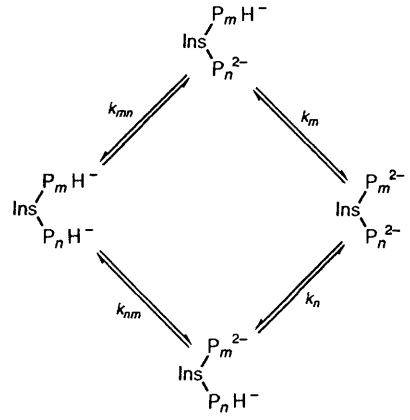
$$H_{y-1}L^{(n-y+1)-} + H^+ \rightleftharpoons H_yL^{(n-y)-}$$

holds with an equilibrium constant K_y. The constants β_y and K_y are usually determined by potentiometric methods, and in simple cases by NMR measurements. For instance, for an inositol monophosphate, K_1 can easily be derived from eqn. (5).

$$f_{i,p} = \bar{p} = \frac{K_1 \times [H^+]}{1 + K_1 \times [H^+]} \quad (5)$$

However, for inositol polyphosphates, as for other polyprotic acids, these macroscopic constants are most often composites of microscopic constants reflecting the behaviour of each individual phosphate group since their basicity can be of the same order of magnitude. Numerous papers have reported the determination of microscopic constants of diprotic acids, mainly in terms of dissociation constants.[25–28]

The protonation scheme of an inositol diphosphate can be described as depicted in Scheme 1. The subscripts m and n refer



Scheme 1

to the position of the phosphate on the inositol ring. k_m, k_n, k_{mn} and k_{nm} are micro protonation constants. It should be noted that in our study only the first protonation step is considered for each phosphate since the second basicity of the phosphates is very weak.

The macro- and micro-constants are related by eqns. (6)–(8).

$$K_1 = k_m + k_n \quad (6)$$

$$\frac{1}{K_2} = \frac{1}{k_{mn}} + \frac{1}{k_{nm}} \quad (7)$$

$$K_1K_2 = k_m \times k_{mn} = k_n \times k_{nm} \quad (8)$$

For Scheme 1, the protonation fractions $f_{m,p}$ and $f_{n,p}$ can be defined as shown in eqns. (9) and (10).

$$f_{m,p} = \frac{[IP]_{mn}[H_2] + [IP_mH]}{[IP] + [IP_mH] + [IP_nH] + [IP_{mn}H_2]} = \frac{k_{mn}k_m[H]^2 + k_m[H^+]}{k_{mn}k_m[H^+]^2 + (k_m + k_n)[H^+] + 1} \quad (9)$$

$$f_{n,p} = \frac{[IP_{mn}H_2] + [IP_nH]}{[IP] + [IP_mH] + [IP_nH] + [IP_{mn}H_2]} = \frac{k_{mn}k_m[H^+]^2 + k_n[H^+]}{k_{mn}k_m[H^+]^2 + (k_m + k_n)[H^+] + 1} \quad (10)$$

Knowing the values for $f_{m,p}$ and $f_{n,p}$ from NMR experiments and the macro-constants from potentiometry, there are different ways of calculating the micro-constants.[26,27] One of the most suitable methods[27] consists of introducing K_1 into the denominator of eqn. (9) and (10), and then solving these equations to calculate k_m, k_n and $k_{mn} \times k_m$.

For inositol triphosphates, the determination of micro-constants becomes more complicated, since there are seven different protonated forms to be considered instead of three for an inositol diphosphate. At this stage of our investigation this problem has not been fully solved. Nevertheless, the ionization state at a given pH can still be well described by considering the plots of $f_{i,p}$ *versus* pH.

Experimental

Synthesis of the Ligands.—(±)Ins(1,4,5)P_3 and (±)Cyhx-(1,2,4)P_3 were synthesized as previously described.[17,29]

(±)Ins(1,4)P_2 was prepared by phosphorylation of (±)-1,2-4,5-di-*O*-isopropylidene-*myo*-inositol[30] using *N*,*N*-diethyldibenzylphosphoramidite followed by oxidation with *m*CPBA

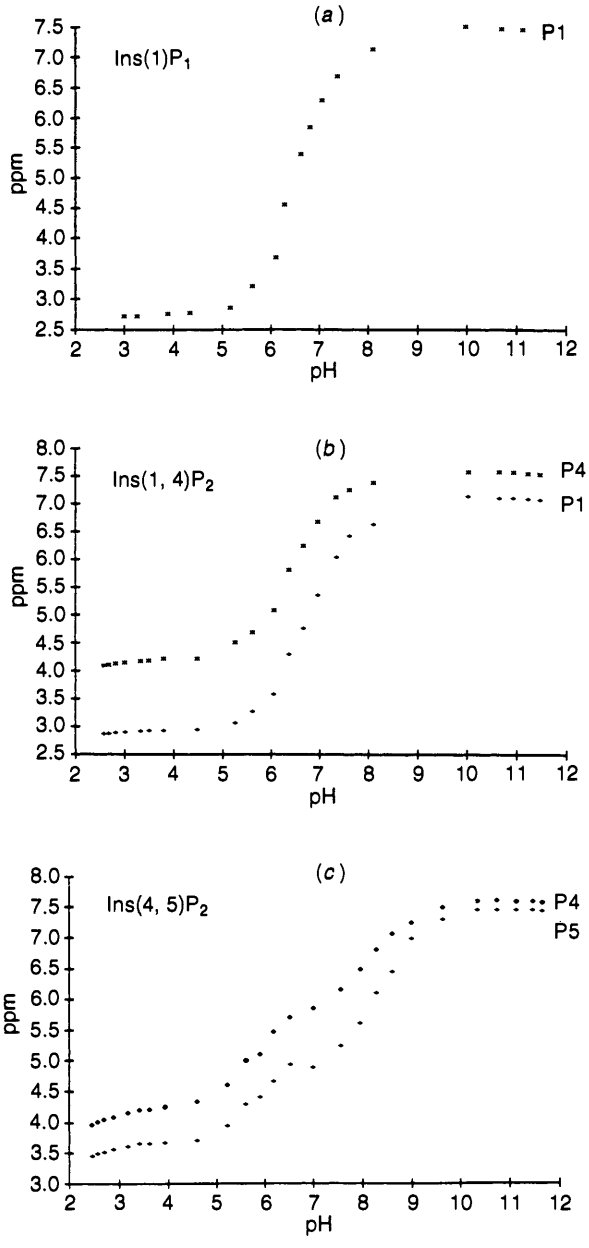


Fig. 1 Chemical shifts δ from ^{31}P NMR titrations as a function of pH: (*a*) Ins(1)P_1; (*b*) Ins(1,4)P_2; and (*c*) Ins(4,5)P_2

according to Perich and Johns[31] (yield 62%). Hydrolysis [methanol–HCl (1 mol dm^{-3}) 9:1; reflux; 40 min], crystallization (yield 87%) and then hydrogenation (H_2, Pd/C 10%; ethanol 95%; 1 atm; 20 °C, 12 h) (yield 46%) gave the Ins(1,4)P_2.

(±)Ins(4,5)P_2 was prepared by phosphorylation of (±)1,2-*O*-isopropylidene-3,6-di-*O*-benzyl-*myo*-inositol[32] in the same manner as Ins(1,4)P_2. Subsequent hydrolysis [methanol–HCl (1 mol dm^{-3}) 9:1; reflux 30 min], crystallization (overall yield 43%) and finally hydrogenation produced Ins(4,5)P_2 with 80% overall yield.

(±)Ins(1)P_1 was obtained starting from (±)1,2:5,6-di-*O*-cyclohexylidene-4-*O*-benzyl-*myo*-inositol[33] which was phosphorylated with 3-diethylamino-1,5-dihydro-2,4,3-benzodiazaphosphepine followed by oxidation with *m*CPBA according to Watanabe *et al.*[34] (yield 95%). The product solubilized in 95% ethanol was hydrogenated and the ketals removed by hydrolysis in acetic acid (2 h, 20 °C) (yield 38%).

All these IPs were stabilized as cyclohexylammonium salts.

Potentiometric Studies and NMR Determinations.—For each experiment, 3 cm^3 of aqueous IP salt was treated on an Amberlyst IRN(H^+) column to convert the cyclohexylammonium salt into its acidic form. The volume of the eluate was adjusted to 5.0 cm^3 in a flask containing 0.5 cm^3 of ^2H_2O and the supporting-electrolyte *i.e.* Et_4NClO_4 for medium 1 (0.1 mol dm^{-3}) and KCl for medium 2 (0.2 mol dm^{-3}). The resulting solution (at a concentration of about 2×10^{-3} mol dm^{-3}), was used the same day for both potentiometric and NMR measurements.

Initially, 2.0 cm^3 of the sample solution were titrated in a 5 cm^3 thermoregulated (25.0 or 37.0 ± 0.1 °C) cell with an adequate base. For medium 1, the titration reactant was tetramethylammonium hydroxide and for medium 2, potassium hydroxide. In all cases, fresh twice-distilled water was used. The electrode and the automatic titration equipment have been previously described.[35] The potentiometric titration allowed the concentration of the IP ($C°_L$) and the total concentration of the acid ($C°_H$) to be determined. Processing of the pH measurements by the specific program GRAPHIPOT[36] yielded the macroscopic protonation constants.

For the NMR titration, the same initial volume of sample solution and the same additions of the base were used. To avoid loss of sample on the glass electrode used for pH determination, the potentiometry pH values previously measured were used.

^{31}P NMR spectra were recorded at 145.785 MHz on a Bruker AM360 Fourier transform spectrometer. Experiments were performed with a standard 10 mm broad band probe head and Wilmad 513-1PP 10 mm sample tubes. Field-frequency lock was achieved using deuterium nuclei present in the sample (10% ^2H_2O, see above). Chemical shifts were measured relative to an external 85% orthophosphoric acid reference (20 176.58 Hz = 0.00 ppm). Samples were spun at 15 Hz, and the sample temperature was regulated to 25.0 or 37.0 ± 0.2 °C by a nitrogen flow, using the standard Bruker temperature control unit. Decoupling of the phosphorus nuclei from protons was achieved by continuous irradiation of the protons with composite pulses centred around 6500 Hz, with an irradiation power of 0.25 W. After a flip pulse of 10 μs duration (corresponding to a 60° flip angle), data were acquired over spectral sweep widths of 5 kHz, and 4 K complex data points (acquisition time 557 ms). Data collection was repeated every 3 s, to obtain a reasonable signal to noise ratio (typically 20 to 3000 transients, depending on the linewidths). Prior to Fourier transformation, the decay signal was zero-filled to 16 K complex data points, and an exponential multiplication (2–3 Hz line broadening) was applied.

Results and Discussion

Assignment of Phosphorus Resonances.—Phosphorus resonances of Ins(1,4,5)P_3 were first assigned by performing phosphorus–proton 2D correlation experiments and comparing the results with previously published chemical shifts.[37–39] The phosphorus resonances of Ins(1,4)P_2 and Cyhx(1,2,4)P_3 were assigned by analogy with those of Ins(1,4,5)P_3. In the case of the latter compound, P1 and P2 have very close chemical shifts and therefore cannot be distinguished unambiguously and their position in the spectra may be interchanged.

Inositol Mono- and Di-phosphates.—In Fig. 1, the ^{31}P NMR titration curves of Ins(1)P_1, Ins(1,4)P_2 and Ins(4,5)P_2 are shown. In Fig. 2, $f_{i,p}$ is plotted against pH for the same ligands, to produce a normalized representation of the protonation process. For the pH range considered (11–2.5), the chemical shift variations correspond to the gain of one proton on each phosphate group, the second basicity of the phosphates being very weak, the second protonation occurs only for pH values

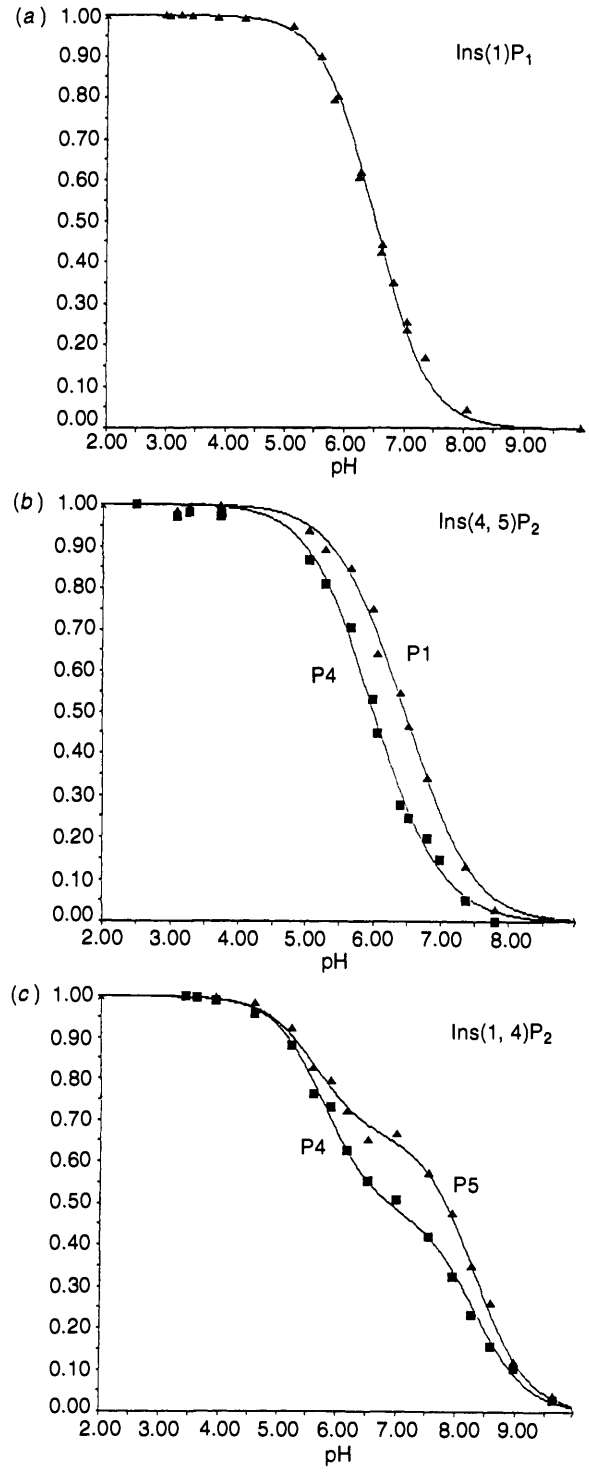


Fig. 2 Protonation fraction $f_{i,p}$ *versus* pH for: (*a*) Ins(1)P_1; (*b*) Ins(1,4)P_2; and (*c*) Ins(4,5)P_2

below 2.5. The titration curve of Ins(1)P_1 resembles those of numerous other monophosphate esters,[24] and can be considered as a good model curve for which the addition of one equivalent of acid affects only the protonation of a single phosphate group. Use of eqn. (5), which describes the theoretical behaviour of a single protonation step, provides a value of 6.50 ± 0.04 for log K_1, and the curve recalculated from this result superimposes very well with the experimental data points [Fig. 2(*a*)].

For Ins(1,4)P_2 and Ins(4,5)P_2 the shape of the titration curves are clearly different. Whereas for Ins(1,4)P_2 the curves retain a monophasic appearance, they are for Ins(4,5)P_2 distinctly biphasic. Such differences reflect the marked influence of the position of the phosphates on the inositol ring, which determines the possible interaction between phosphate groups. In this case, the calculation of microscopic protonation constants according to the equations given above [eqns. (6)–(10)] becomes particularly useful since it provides a direct access to the quantification of the ionization state of each group. The logarithms of the potentiometrically determined macro-constants, log K_1 and log K_1K_2, are for Ins(1,4)P_2 6.85 ± 0.02 and 12.84 ± 0.02 respectively, and for Ins(4,5)P_2 8.33 ± 0.03 and 14.30 ± 0.03. Solving eqns. (9) and (10) after introducing K_1 leads to the values of the micro-constants. For Ins(1,4)P_2, log k_1 = 6.59 ± 0.06, log k_4 = 6.22 ± 0.1, log k_{14} = 6.22 ± 0.15 and log k_{41} = 6.45 ± 0.10. From these results k_1/k_4 can be calculated to have a value of 2.30. This is an indication of the lower basicity of the P4 phosphate, presumably due to a greater probability of hydrogen bonding with two neighbouring equatorial hydroxy groups as compared to the situation with one equatorial and one axial hydroxy group. It can also be noted that log k_1 is close to the log K_1 of Ins(1)P_1, indicating similar acid–basic properties for the two phosphate groups in the same position, and showing the relative independence of the phosphates in position 1 and 4, in a *para* position on the ring. In addition, log k_{14} and log k_{41} have values close and complementary to those of log k_1 and log k_4 (log $k_1 \simeq$ log k_{41} and log k_4 = log k_{14}). This confirms the equivalence and independence of both protonation sites. For Ins(4,5)P_2, log k_4 = 8.01 ± 0.07, log k_5 = 8.15 ± 0.01, log $k_{45} \simeq$ 6.09 ± 0.05 and log k_{54} = 5.82 ± 0.05. These values, far from those of Ins(1,4)P_2, are characteristic of two vicinal phosphate groups in equatorial positions. Indeed, the high log k_4 and log k_5 values clearly indicate that the first proton is shared by both phosphates, even if the P5 phosphate shows a slightly greater basicity (k_4/k_5 = 0.72). The approach and binding of the second proton is largely hindered (log k_{45} and log $k_{54} \ll$ log k_4 and log k_5) by the electrostatic repulsion of the first proton shared by both phosphate groups.

Inositol Triphosphate and Related Compounds.—Fig. 3 displays the chemical shifts against pH for the two triphosphorylated compounds, *i.e.* Cyhx(1,2,4)P_3 and Ins(1,4,5)P_3 in two different media. Fig. 4 shows the $f_{i,p}$ curves for these compounds, determined in medium 1. It appears from Figs. 3 and 4 that the curves of Ins(1,4,5)P_3 and its dehydroxylated analogue Cyhx(1,2,4)P_3, markedly differ, reflecting the great importance of the hydroxy groups in the protonation process of these molecules.

The curves of Cyhx(1,2,4)P_3 [Figs. 3(*a*) and 4(*a*)] are easy to interpret in view of the results for the inositol biphosphates. The logarithms of the stepwise macro-constants issued from the potentiometric determination are log K_1 = 9.47 ± 0.05, log K_2 = 7.67 ± 0.01 and log K_3 = 6.66 ± 0.02. Apparently, the first and the third constants apply to the two vicinal phosphates P1 and P2 and the second concerns exclusively the P4 phosphate. If there are no interactions between the phosphate P4 and the phosphates P1 and P2, the case of Cyhx(1,2,4)P_3 can be treated by considering that the curves result from the superimposition of those of two interacting neighbouring phosphates with the curve of an independent monophosphate. As can be seen from Fig. 4(*a*), the experimental curves for P1 and P2 are fitted very well, using eqns. (9) and (10), and give micro-constants log k_1 = 9.14 ± 0.02, log k_{12} = 7.06 ± 0.08, log k_2 = 9.17 ± 0.02 and log k_{21} = 7.01 ± 0.06. These constants show that unlike for Ins(4,5)P_2, the first and third protons of the molecule are similarly shared by the two phosphate groups. Moreover, the fit of the P4 curve by eqn. (5) remains satisfactory and gives for this group log K = 7.53 ± 0.06. It can thus be concluded that the assumption previously made holds true for Cyhx(1,2,4)P_3.

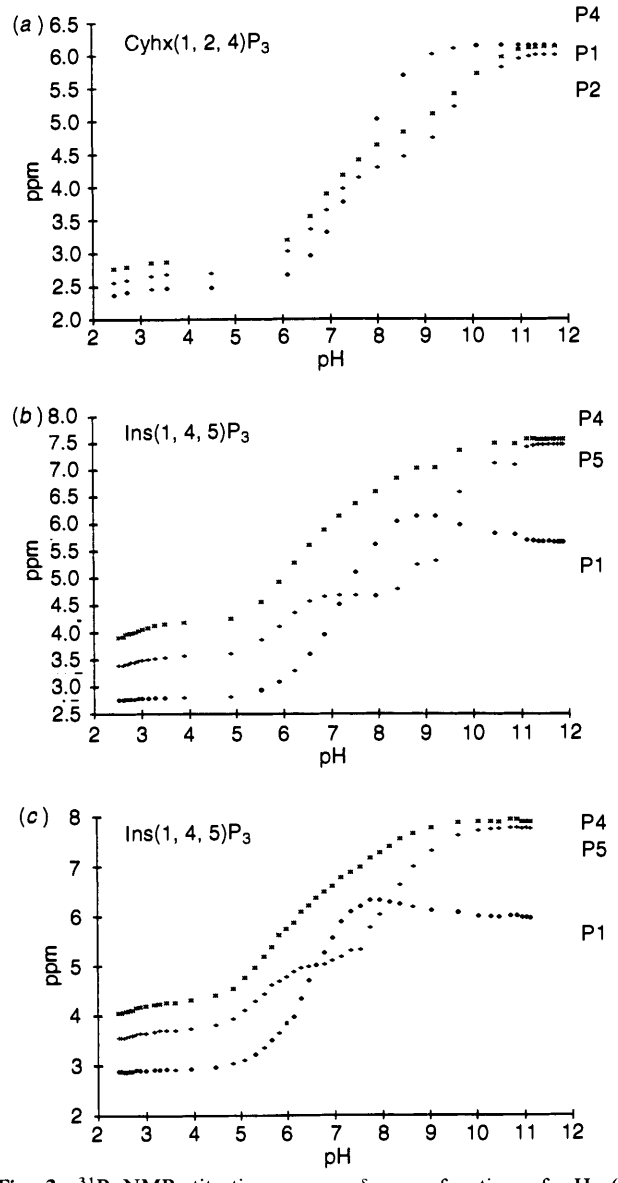


Fig. 3 ^{31}P NMR titration curves: δ as a function of pH. (*a*) Cyhx(1,2,4)P_3; (*b*) Ins(1,4,5)P_3 in Et_4NClO_4 0.1 mol dm^{-3} at 25 °C; and (*c*) Ins(1,4,5)P_3 in KCl 0.2 mol dm^{-3} at 37 °C.

Table 1 Chemical shifts of the monoanionic ($\delta_{i,p}$) and dianionic ($\delta_{i,d}$) forms of the inositol phosphates and derivatives under study

$\delta_{i,p\,ord}$	Ins(1)P_1	Ins(1,4)P_2	Ins(4,5)P_2	Ins(1,4,5)P_3	C(1,2,4)P_3
δ_{1p}	2.73	2.86		2.78	2.40
δ_{1d}	7.50	7.10		5.66	6.15
δ_{4p}		4.10	4.20	4.18	2.80
δ_{4d}		7.56	7.60	7.57	6.12
δ_{5p}			3.65	3.55	2.60
δ_{5d}			7.45	7.45	6.00

The titration curves of Ins(1,4,5)P_3 [Figs. 3(*b*, *c*)] appear to be more complicated, due to the stronger interaction between the different phosphates, allowed by the presence of the hydroxy groups. Going from high to low pH values [Fig. 3(*c*)], the protonation process of all the phosphates is far from that of a simple monoester. The protonation sequence involves at each step several phosphate groups. With the addition of a first equivalent of protons (from $\bar{p} = 0$ to 1), the phosphate P5 undergoes a large upfield shift, corresponding to an approximate protonation of about 70% for this group. The same tendency is observed for the phosphate P4, even though the protonation fraction remains much lower (about 23%). Surprisingly, the chemical shift of P1 first goes downfield and seems involved in a deprotonation process and then reverts upfield in the expected direction. The addition of a second equivalent of protons (from $\bar{p} = 1$ to 2) only affects phosphates P4 and P1. The former is protonated to about 50% whereas the latter is protonated to approximately 80%. For the phosphate P5, the protonation state remains strictly unchanged. Finally, the third equivalent of protons is shared by all three phosphates P1 (20%), P4 (50%), and P5 (30%).

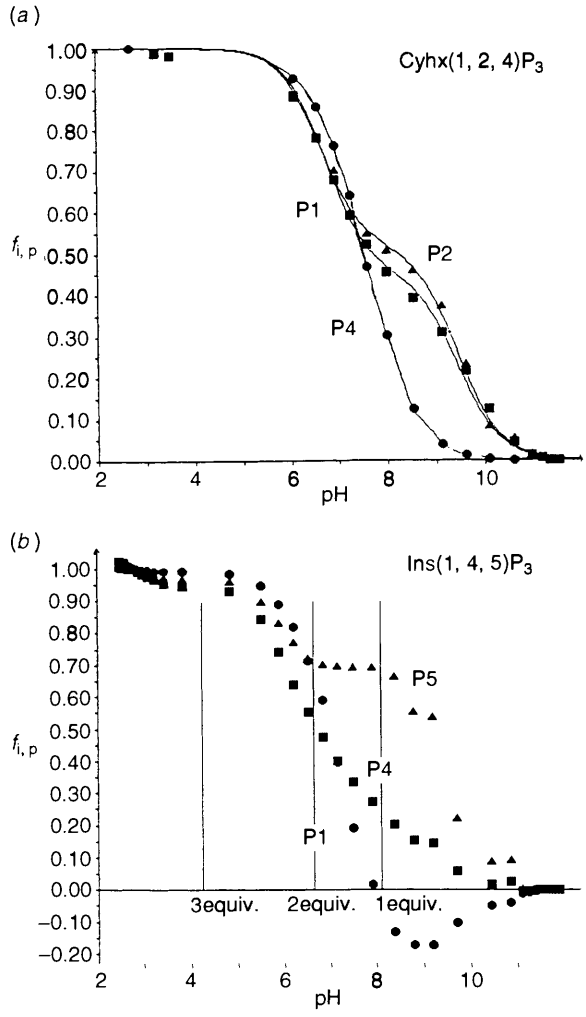


Fig. 4 Protonation fraction $f_{i,p}$ *versus* pH for: (*a*) Cyhx(1,2,4)P_3; (*b*) Ins(1,4,5)P_3 in Et_4NClO_4 0.1 mol dm^{-3} at 25 °C. In (*a*), the vertical lines correspond to the theoretical addition of 1–3 equiv. protons.

The protonation of Ins(1,4,5)P3 can also be followed on the $f_{i,p} = f(pH)$ curves [Fig. 4(*b*), the vertical lines corresponding, right to left, to the theoretical addition of 1 to 3 equivalents of protons]. Only the curves for P4 and P5 rigorously describe the binding of protons to phosphate groups. For P1, due to the initial downfield shift, the $f_{1,p}$ values are negative at high pH, and this has no physicochemical meaning.

A. M. White *et al.*[37] recently reported the NMR titration curves for Ins(1,4,5)P_3. Although the medium is different and contains alkali-metal cations, the general shape of the curves is the same. Nevertheless, they report no downfield shift of P1, as was observed in our experiments.

Of particular interest is the comparison of the chemical shifts of the monoanionic and dianionic forms of the phosphates at a

Table 2 Variations of the $^3J_{HCOP}$ coupling constant for P1 *versus* pH

pH	$^3J_{HCOP}$
2.39	9.0
6.88	6.9
8.86	5.7
9.5[a]	3.4
11.64	<2.0

[a] See ref. 42.

given position on the ring for the studied compounds (Table 1). For all the IP [except for Ins(1,4,5)P_3], regardless of the number and the position of the phosphates, the chemical shifts of the deprotonated forms are approximately the same. In contrast, the chemical shifts of the monoprotonated forms remain surprisingly constant for phosphates at the same position, but differ markedly from one position to another. Thus, it seems that, at least for monoanionic phosphates, the presence of vicinal hydroxy groups plays a far more important role than the number of neighbouring phosphates.

The influence of an hydroxy group interacting with a phosphate group was previously reported for ribonucleotides and deoxyribonucleotides.[24,40] It has been shown that the presence of a hydroxy group results in a 0.4–0.5 ppm downfield shift of the phosphate resonance signal, whatever the pH. This effect was attributed to hydrogen bonding between the two groups, but also to an electron withdrawing effect of the hydroxy group (through σ bonds) and to solvation effects.[40]

The $\delta_{i,d}$ values for the IPs, as seen, are [except for Ins(1,4,5)P_3], of the same order of magnitude, but notably higher than those for Cyhx(1,2,4)P_3. From these observations, it can be assumed that there is no particular effect of the number and position of the phosphates and hydroxy groups. The difference of about 1.0 to 1.6 ppm is presumably due to the higher deshielding effect of the solvent in the case of the more hydrophilic inositol phosphates, and to the electron-withdrawing effect of the vicinal hydroxy groups.

The co-operation between the phosphate and the hydroxy groups appears particularly marked in the case of Ins(1,4,5)P_3.

At high pH (above 11), Ins(1,4,5)P_3 is totally deprotonated. Each phosphate bears two negative charges. These highly charged groups repel each other to minimize the charge interactions. These respulsions for the phosphates P4 and P5 tend to bring them into the proximity of the hydroxy groups in positions 3 and 6 respectively, which, due to the polarization of the O–H bond, enable some stabilization for the phosphates by hydrogen bonding. The phosphate P1 itself comes near the hydroxy in position 2 and stays far away from the hydroxy in position 6, due to the presence of P5 near this hydroxy group. In such a conformation, all the phosphates are stabilized by a neighbouring hydroxy group with the subtle difference that P1 establishes its interaction with the hydroxy function in position 2 (OH2) in a *cis* configuration, while the two other phosphates have to establish their interaction in a *trans* configuration. This difference in the individual configurations accounts for the shielding of P1 as compared to P4 or P5, and thus its lower chemical shift. P1 appears as if it were partially reprotonated in this configuration (in the chemistry of inositol phosphates, the *cis* configuration between P1 and OH2 may form a cyclic phosphate whereas a *trans* junction has never been reported to generate an analogous cyclic phosphate for inositol phosphates).

It is also worth noting that, at pH above 11, the $^3J_{HCOP}$ coupling constant is less than 2 Hz, which, if we apply the Karplus-like relation established by Haasnoot, corresponds to a dihedral angle of about 90°.[41] Such an angle is consistent with the above mentioned conformation. Our interpretation differs from that proposed by Lindon,[38] who, observing a coupling constant of 3.4 Hz at pH 9.5 (corresponding to a dihedral angle of about 50°), rules out the possibility of an interaction between P1 and OH2. (We shall discuss the discrepancy concerning this $^3J_{HCOP}$ coupling constant later.)

When the pH decreases, a first proton seems to be shared on both P4 and P5. The proton associates the two phosphates in such a conformation that the hydroxy OH3 and OH6 are disengaged. As the first protonation occurs, a deshielding effect is observed, concomitant to an increase of the $^3J_{HCOP}$ coupling constant for P1. These observations can be interpreted as the progressive disengagement of OH6, and, as a consequence, P1 is able to flip more and more freely and rapidly between positions 2 and 6. The coupling constant varies from less than 2 Hz at pH 11.6 to 6.9 Hz at pH 6.9. This latter value can be considered to be an average for the oscillating system, if we suppose rapid dynamics. It is interesting to notice that the coupling constant described by Lindon with a rigid conformation hypothesis (3.4 Hz at pH 9.5) can be included in the variations we have observed against pH (Table 2).

If the pH keeps on decreasing, the phosphate P1 gains a proton, and finally the pair of phosphates P4 and P5 capture a second proton, leading to a triprotonated species, each phosphate bearing one proton. At this step the chemical shift for P1 becomes very different from the chemical shift observed for the other phosphates, P4 and P5. This phenomenon appears for all P1 (or its enantiotopomers) of various inositol phosphates (Table 1). This abnormal value could, again, be due to the particular orientation of the hydroxy OH2, causing a difference in solvation of this part of the molecule.

Concerning the monoanionic phosphates, the $\delta_{i,p}$ values for Cyhx(1,2,4)P_3 (2.60 ± 0.20 ppm) may be taken as a reference for the chemical shift of a monoprotonated phosphate group which experiences no hydroxy interaction. The $\delta_{1,p}$ for all the mono-, di- and tri-phosphoinositols under study are very close to this value. Thus, it can be supposed that the monoprotonated phosphate in position 1 behaves independently, not influenced at all by the presence of the vicinal hydroxy groups. The $\delta_{4,p}$ values of the IPs show a downfield shift of about 1.2 to 1.4 ppm with respect to the corresponding $\delta_{1,p}$. Such a high deshielding effect can only be the result of a strong hydrogen bond, between the hydrogen atom of the phosphate and the free electron pairs of the vicinal hydroxy oxygen at position 3. A cooperative effect of the axial OH at 2 can, in addition, be expected. In this respect, the most likely conformation is that involving a *gauche* orientation,[38] with a P–O–C–H torsion angle of about 50–60°. For Ins(1,4)P_2, the value of the J_{H4-P} coupling constant at pH 2.5, *i.e.* for the monoprotonated phosphates (3.5 Hz), is consistent with such an arrangement. The $\delta_{5,p}$ values lie between the observed values for $\delta_{1,p}$ and $\delta_{4,p}$, typically reflecting an averaged behaviour. The predominant rotamer should still have a *gauche* orientation, due to the deflection of the phosphate towards the OH6. Such an interpretation is in good agreement with the conclusions of J. C. Lindon *et al.*[38] who studied the molecular conformation of Ins(1,4,5)P_3 based on ^1H, ^{13}C and ^{31}P NMR spectra.

The titration curves of Ins(1,4,5)P_3 determined in medium 2, *i.e.* in the presence of 0.2 mol dm^{-3} potassium chloride [Fig. 3(*c*)], retain the same general shape as in the absence of the alkali-metal cations. In particular, the initial downfield shift of P1 is still observed, and it is noteworthy that the shifts revert upfield near the physiological pH. The main difference is a large shift of the end points of the various titration curves at lower pH values. This can be explained by the strong competition between the protons and the potassium cations for the binding sites.

In a previous study,[17] we reported the good agreement

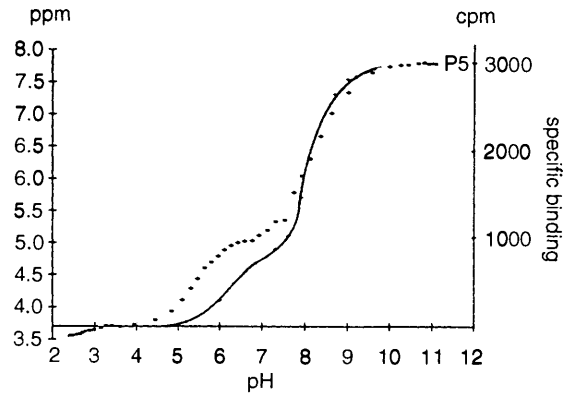


Fig. 5 Titration curve $\delta(^{31}P) = f(pH)$ for the phosphate P5 of Ins(1,4,5)P_3 in 0.2 mol dm^{-3} KCl at 37 °C and binding data from ref. 16

between the fully deprotonated distribution curve and the specific binding data *versus* pH of Ins(1,4,5)P_3 to brain membrane receptors published by P. F. Worley *et al.*[16] In Fig. 5, the same binding data are shown superimposed on the P5 titration curve obtained in medium 2. Both curves coincide quite well at pH values above 7.5, and in both cases a shoulder exists. This seems to prove that Ins(1,4,5)P_3 binds to the receptor with the phosphate at position 5, or that at least P5 plays an important role in the binding mechanism.

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