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Conformational preferences and intramolecular interactions of myo-inositol hexakisphosphoric acid by ^{1}H and ^{31}P NMR studies

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ABSTRACT: The long-standing issue of the conformational change of myo-inositol hexakisphosphoric acid (H_{12}inhp), commonly called phytic acid, was resolved by low-temperature pH* studies and NMR spectroscopy. Low-temperature experiments on phytic acid, in a suitable mixed solvent and in the appropriate pH* range ($10.0 < pH^* < 11.3$), allowed the detection of separate NMR lines corresponding to two conformers. Chemical shift variations as a function of pH* reveal that at pH* >10.2, the mono-, di- and triprotonated species, and also the entirely deprotonated species, are stabilized in the axial form (five C—O in the axial position and one C—O in the equatorial position) and at pH* <11.3, in the equatorial form for other protonated species of phytic acid (one C—O in the axial position and five C—O in the equatorial position). From the NMR spectra, we conclude that the structural change is triggered by the ninth acid dissociation of phytic acid. We suggest that the stabilization of the axial and equatorial conformations is due partly to the presence of C—H $\cdots$ O—P through space interactions, and partly to trans-annular hydrogen bonding between all the phosphate groups. Copyright © 1999 John Wiley & Sons, Ltd.

KEYWORDS: myo-inositol hexakisphosphoric acid; conformation; intramolecular interactions; ^{1}H NMR; ^{31}P NMR

INTRODUCTION

Phytic acid is an interesting physiologically active compound.[1] It constitutes 1–3% of most plant seeds[2] and usually occurs as a mixed calcium–magnesium–potassium salt (phytin) in discrete regions of the seeds.[3] Its tremendous chelating potential and its effects on the bioavailability of metallic cations have been the subject of several papers.[4–6] Numerous other studies have also shown its usefulness as an antioxidant and scanning agent.[7,8]

In order to understand its biochemical roles, we have to determine the protonation equilibrium constants and the conformational changes with pH. It has been postulated that the preference of various phytate anions for the axial conformations is due to decreased electrostatic repulsion between negatively charged vicinal equatorial phosphates in the equatorial conformations, and also to stabilization of the sterically hindered axial conformations by hydrogen bonding between the *syn*-oriented phosphates.[9,10]

In this paper, we describe low-temperature investigations of conformational dynamics and proton transfer phenomena. Our strategy was based on the use of tetrabutylammonium ions as counterions to obtain the dissolution of the phytate anions at low temperature. The ^{31}P NMR titration curves indicate that the deprotonation processes of phytic acid are complex. These processes are such that they change markedly the electron density on several phosphate groups, leading to ‘anomalous’ NMR curves. The number of inflections for the phosphates supports the contention that extensive proton sharing occurs in the axial and equatorial conformations.

We report NMR spectroscopic evidence for intramolecular C— H $\cdots$ O through-space interactions for phytic acid in solution; evidence provided by the electrostatic perturbation observed on the chemical shifts of some C—H methine protons during the deprotonation processes.

EXPERIMENTAL

Hydrated myo-inositol hexakisphosphoric acid sodium salt (Na_{12}Inhp), purchased from Sigma Chemical, was converted into phytic acid (H_{12}Inhp) as described previously.[11] For low-temperature (256 K) NMR experi-

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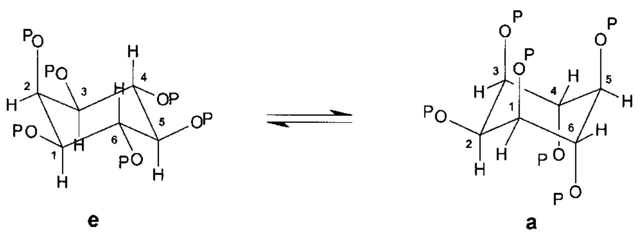

Figure 1. Equatorial (**e**) and axial (**a**) conformations of phytic acid. P = —PO_3H_2, —PO_3H^- or —PO_3^{2-} depending on pH

ments, lyophilized phytic acid (3×10^{-2} mol l^{-1}) was used in CD_3OD–D_2O mixed solvent, for a methanol–water composition of 30:70 (v/v). Self-association of phytic acid was checked with dilution experiments and was found to be negligible in the concentration range $0.5 \times 10^{-2} - 4 \times 10^{-2}$ mol l^{-1} from ^1H NMR chemical shift measurements.

The ^1H and ^{31}P NMR spectra were recorded on a Bruker ARX200 Fourier transform spectrometer operating at 200 MHz on ^1H and 80.0 MHz on ^{31}P. ^{31}P and ^1H chemical shifts were measured relatively to trimethylphosphate and methanol, respectively, as internal references. NMR experiments at 256 K were conducted under instrument control and the temperature was controlled within ±1 °C.

A Metrohm E605 pH-meter coupled with a combined glass electrode was used for pH* adjustments under an argon atmosphere. The electrode was calibrated at 256 K by means of standard base and acid (NaOH and HCl) in

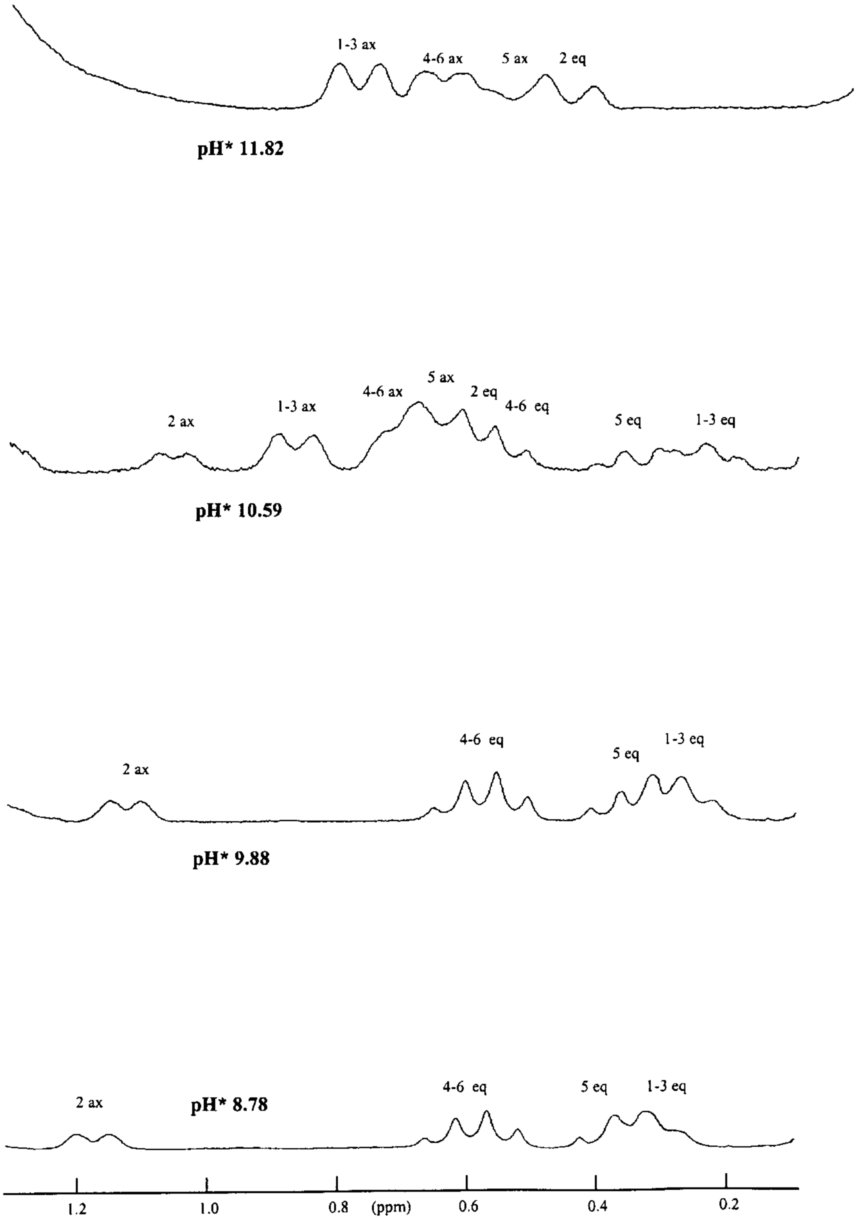

Figure 2. ^1H selected NMR spectra at different pH* and $T = 256$ K

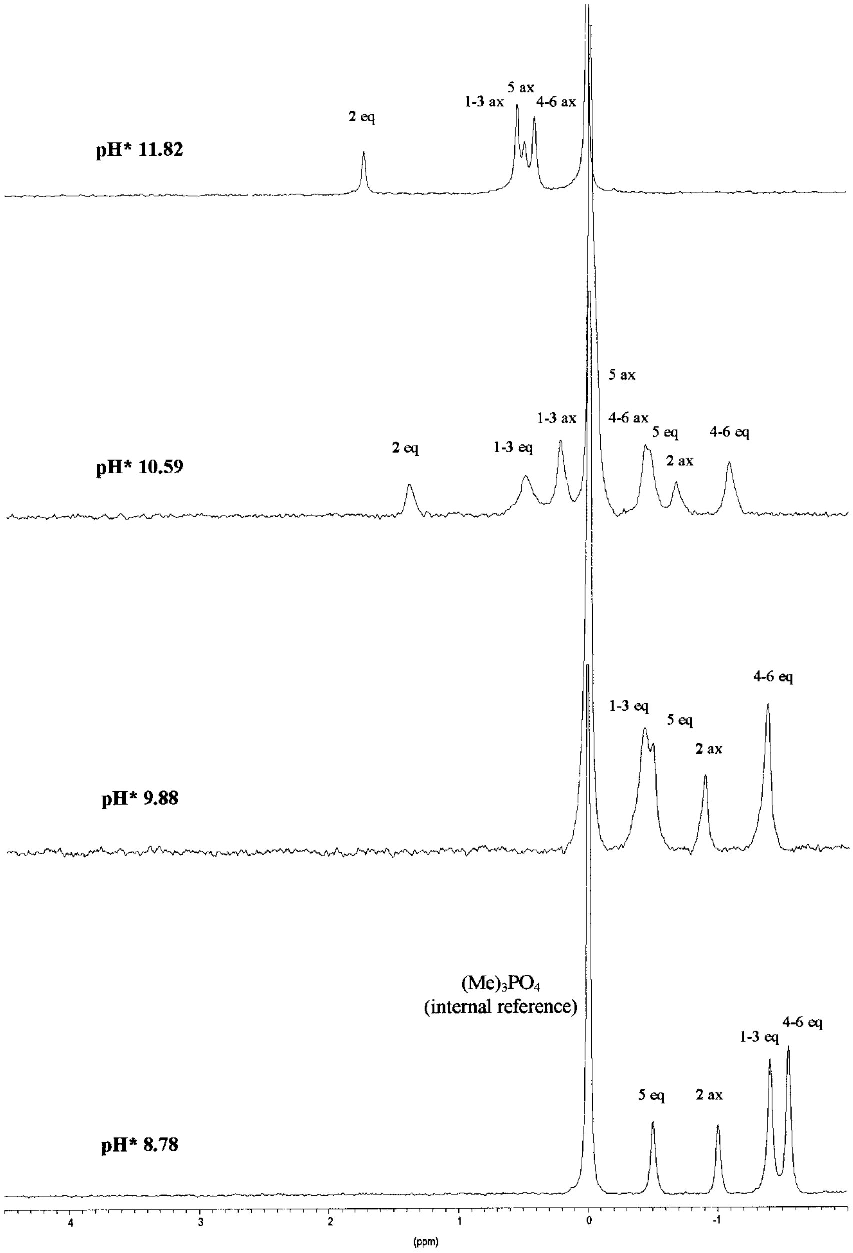

Figure 3. ^{31}P–{^{1}H} selected NMR spectra at different pH* and $T = 256$ K

$CH_3OH–H_2O$ mixed solvent (30:70, v/v).[12] The pK_s (= 15.5) of the mixed solvent was determined graphically by plotting, at 256 K, pH* and p(OH*) vs the mV meter reading from three solutions of standard strong acid (HCl) and three solutions of standard strong base (NaOH), in mixed solvent. No correction was made to the pH for isotopic effect. pH* is the value read on the pH-meter when the electrode is placed in the deuterated mixed solvent.

Assignments of ^{1}H and ^{31}P resonances of phytic acid in axial conformations were made from a two-dimensional ^{1}H–^{1}H double quantum filtered correlation experiment (DQFCOSY) performed by Barrientos and murthy[13] and a two-dimensional ^{1}H–^{31}P heteronuclear shift correlation experiment performed by ourselves, respectively.

RESULTS AND DISCUSSION

Conformational analysis

The conformation of phytic acid was investigated at 256 K by NMR spectroscopy over the pH* range 9.0–13.0 at intervals of about 0.2 pH units. Interconversion between an axial and an equatorial conformation of phytate anions (Fig. 1) at a slow rate compared with the NMR time-scale is strongly evidenced by the ^{1}H and ^{31}P NMR spectra in Figs 2 and 3. In the pH* range 10.0–11.3, the ^{1}H NMR spectra consist of two sets of signals with distinct splitting patterns corresponding to axial and equatorial conformers. The pH* increase from 10.0 to 11.3 is accompanied by changes in the ^{1}H NMR

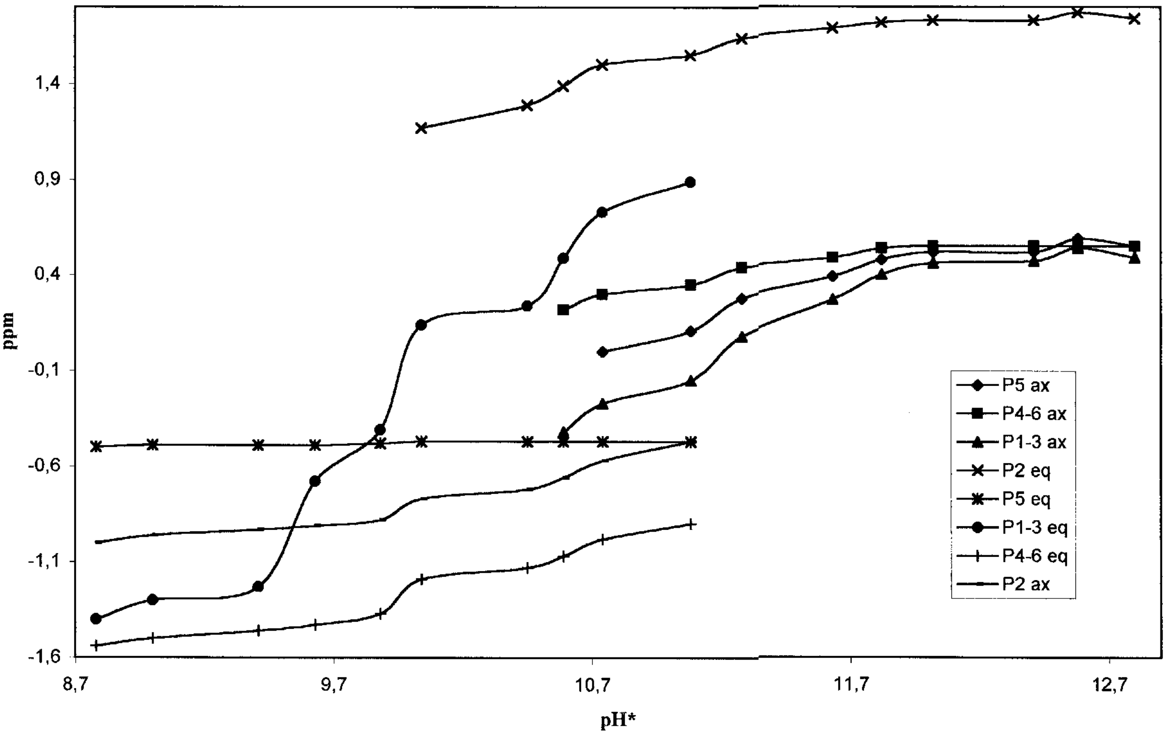

Figure 4. $^{31}P-\{^1H\}$ NMR titration curves of phytic acid at 256 K (17 spectra, recorded in the pH* range 9–13)

spectrum. We observe a decrease in intensity of methine signals corresponding to equatorial conformations and an increase of methine signals corresponding to axial conformations.

Inspection of the values of the vicinal coupling constants from ^3J(HCOP) and ^3J(H–H) provides conformational information.[11] The vicinal coupling constants from the $^1H-\{^{31}P\}$ NMR spectra are affected only by dihedral angles between vicinal protons. The coupling constant is 2–3 Hz when the relationship is axial–equatorial [$^3J(H_{ax}-H_{eq})$] and 8–10 Hz when it is axial–axial [$^3J(H_{ax}-H_{ax})$]. The value of ^3J(HCOP) is 9–11 Hz when phytic acid is in an equatorial conformation and 11–13 Hz when the conformation is axial.[11] According to Lankhorst *et al.*,[14] ^3J(HCOP) depends on the dihedral angle ϕ = H—C—O—P, following Karplus-type curves based on the equations

$$^3J(\text{P–O–C–H}) = 18.1\cos^2\phi - 4.8\cos\phi \quad \text{for } 0 < \phi < 90^\circ \qquad (1)$$

$$^3J(\text{P–O–C–H}) = 15.3\cos^2\phi - 6.1\cos\phi + 1.6 \quad \text{for } 90 < \phi < 180^\circ \qquad (2)$$

^{31}P chemical shifts and ^3J(HCOP) coupling constants may be influenced by pH*, hydrogen bonds (which may induce an internal rotation of phosphate groups around C—O single bonds) and ring conformational changes.

The first assignment of ^1H resonances was made by Johansson *et al.*[15] at pH* 5.5 and confirmed by us [11] at pD 0.68. While $^3J(H_{ax}-H_{eq})$ is well resolved at 25 °C,[11] the one-dimensional ^1H NMR spectra recorded at −17 °C in the pH* range 9–13 do not exhibit $^3J(H_{ax}-H_{eq})$ coupling patterns, the maximum value of any splitting (2.5 Hz) due to these couplings being of the order of the linewidths. The ^1H NMR spectrum at pH* 9.88 recorded at −17 °C (Fig. 2) consists of three groups of signals: two quartets at δ 0.58 and 0.34 ppm, one triplet at 0.28 ppm and one broad doublet at δ 1.13 ppm. They have been assigned to the magnetically equivalent $H_4 + H_6$ (notation $H_{4,6}$) and H_5 protons (the two quartets), $H_{1,3}$ (the triplet) and H_2 (the doublet), respectively, from two-dimensional $^1H-^1H$ homonuclear shift correlation experiments and ^1H spin systems analysis. The splitting of the H_2 signal into one broad doublet is due to a large coupling (9.3 Hz) with P. The splitting of magnetically equivalent $H_{4,6}$ protons into a quartet is due to couplings of 9.3 Hz with vicinal protons [$^3J(H_{ax}-H_{ax})$] and of 9.5 Hz with phosphorus ($^3J_{H,P}$). H_5 is split into a quartet due to couplings of 8.6 Hz with vicinal protons [$^3J(H_{ax}-H_{ax})$] and of 10.3 Hz with phosphorus ($^3J_{H,P}$). The magnetically equivalent H_1 and H_3 protons are split into a triplet [$^3J(H_{ax}-H_{ax}) \approx {}^3J(\text{HCOP}) = 9.2$ Hz]. The $^1H-\{^{31}P\}$ NMR spectrum at pH* 9.88 consists of a broad singlet (H_2), two triplets ($H_{4,6}$ and H_5) and a doublet ($H_{1,3}$). We can infer that the splitting pattern is consistent with the 1ax/5eq structure in which C_2—O is oriented in an axial position and C_1—O, C_3—O, C_4—O, C_6—O and C_5—O in equatorial positions. The ^1H NMR spectrum recorded at pH*

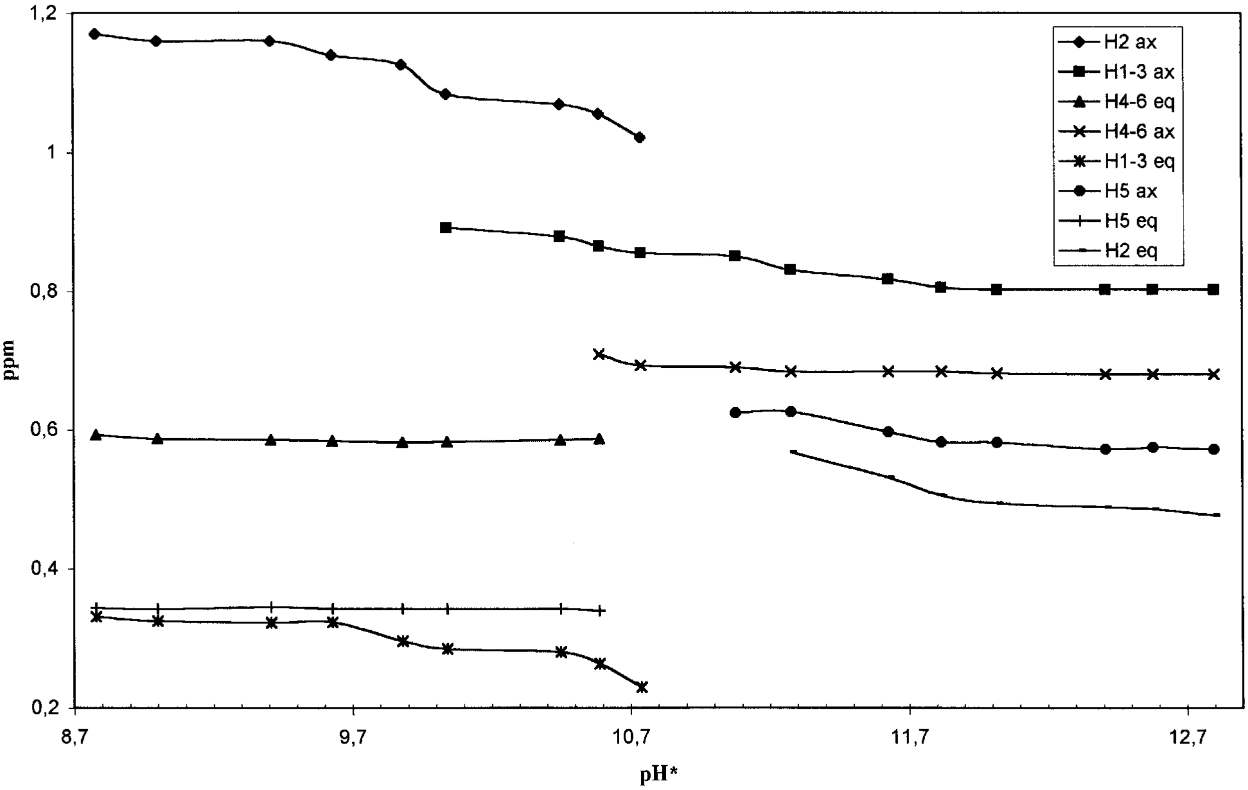

Figure 5. ^{1}H NMR titration curves of phytic acid at 256 K in the pH* range 9–13

11.82 (Fig. 2) consists of four resolved signals, all broad doublets here but appearing as singlets in the ^{1}H–{^{31}P} NMR spectrum. Only one large coupling with phosphorus is observed for each proton. The presence of broad doublets suggests one large coupling with P and some small couplings with vicinal protons (*ca* 2–3 Hz). Similar spectra with the same coupling patterns, recorded in the pH* range 11.3–13.0, indicate that phytate anions present over the pH* range 11.3–13.0 adopt axial conformations.

^{31}P NMR spectra

Some selected spectra at representative pH* values are shown in Fig. 3 and a plot of pH* vs δ_p in Fig. 4. The solution pH was increased from pH* 9.0 to 13.0 by addition of tetrabutylammonium hydroxide (NBu_4OD). We note that whatever the pH* and the conformation, phosphorus P_1 (on C_1) and P_3 (on C_3) (notation $P_{1,3}$) on the one hand, and P_4 (on C_4) and P_6 (on C_6) (notation $P_{4,6}$) on the other, are still magnetically equivalent. Figure 3 shows the ^{31}P–{^{1}H} spectrum of phytic acid at pH* 10.59. Distinct phosphorus resonances, all singlets, corresponding to two conformers are observed. According to our data, two phytate anions are present as an interconverting mixture of axial and equatorial conformations in the pH* range 10.0–11.3. When the pH* increases from 10.0 to 11.3, a decrease in the intensity of equatorial signals and an increase in that of axial signals is observed. Phytate anions are present in axial conformations when the pH* is above 11.3 and in equatorial conformations when the pH* is below 10.0.

Except for P_5 and $P_{1,3}$, the ^{31}P NMR titration curves of P_2 and $P_{4,6}$ in phytic acid (Fig. 4) in equatorial conformations are similar to some extent in their general shape and also in the position of their inflection points (apparent pK_as). We can note that 'equatorial P_5 phosphorus' is insensitive to the deprotonation process, whereas the $P_{1,3}$ phosphorus which display three large inflections at 9.6, 10.0, 10.6 are strongly pH* dependent. NMR curves of $P_{4,6}$ and P_2, which both display two small inflections at 10.0, 10.6, are also pH* dependent in the pH* range 9.0–11.3, but are affected by the loss of protons to a smaller extent than $P_{1,3}$. Two inflection points occurring at the same pH* values, for $P_{1,3}$, P_2 and $P_{4,6}$, respectively, indicate that over the pH* range 9.9–11.1 the intramolecular interactions involve a delocalization of protons between phosphate groups linked by the same hydrogen bonds. Noticeable changes are observed on hydrogen bonds over the pH* range 9.0–10.0. We can observe that only $P_{1,3}$ (see Fig. 4) display an inflection at pH* 9.6. This indicates that P_1 and P_3 are coupled together through the same hydrogen bond. As P_1 and P_3 are still magnetically equivalent, the hydrogen is positioned half-way between two identical proton-acceptor oxygen atoms (one oxygen being linked to P_1

and the other to P_3), or there are two equally populated tautomeric forms, in fast equilibrium so that the average position of the hydrogen is still half-way between the two identical acceptors.[16]

The ^{31}P NMR titration curves of phytic acid in the axial conformations are very similar in their general shapes. Chemical shifts of ^{31}P nuclei are all affected by acid-dissociation processes. We can see (Fig. 4) that the downfield shifts undergone by P_2, $P_{1,3}$, $P_{4,6}$ and P_5 all display inflection points (apparent pK_as) at the same pH* values, 10.6, 11.2 and 11.6. It must be pointed out that P_2, $P_{1,3}$, $P_{4,6}$ and P_5 all behave as three-proton releasing groups. The number of inflections supports the view that extensive proton sharing exists in the various phytate anions with a 5-ax/1-eq structure. Apparent pK_a obtained from inflection points show that the totally deprotonated species and also the mono-, di- and triprotonated species are stabilized in 5-ax/1-eq conformations. Our results suggest that the conformational change is triggered by the ninth acid dissociation of phytic acid.

We conclude that for pH* < 10.0, phytic acid (H_{12}inhp) and the deprotonated species $H_{(12-y)}\text{inhp}^{y-}$ ($y = 1, 2, \cdots, 9$) are present in equatorial conformations. For pH* >11.3, deprotonated species $H_{(12-y)}\text{inhp}^{y-}$ ($y = 10, 11, 12$) of phytic acid are present in axial conformations, and for $10.0 \leq \text{pH*} \leq 11.3$ there is a dynamic exchange process, slow on the NMR time-scale, between equatorial $H_4\text{inhp}^{8-}$ and axial $H_3\text{inhp}^{9-}$ species.

^{1}H NMR spectra

The ^{1}H NMR spectra at representative pH* values are presented in Fig. 2 and the titration curves [pH* = $f(\delta_H)$] of phytic acid in Fig. 5.

In the pH* range 8.8–10.8 for phytic acid in equatorial conformations, two interesting features are (*i*) the invariance of the chemical shifts of H_5 and $H_{4,6}$ protons during the deprotonation process and (*ii*) the upfield chemical shift variations of H_2 and $H_{1,3}$ protons during the deprotonation processes. We can note that H_2 and $H_{1,3}$ protons display three inflections at the same pH values (9.7, 10.0, 10.6) as the $P_{1,3}$ three inflections. This last result indicates that when deprotonations occur, a through-bond perturbation causes a shielding effect on these methine protons.[17] From pH* 10.0, the chemical shifts of $P_{4,6}$ are pH* dependent, but not those of $H_{4,6}$. We might have expected $H_{4,6}$ and H_2 protons to behave in the same way because of through-bond inductive effects, since experimental titration curves for $P_{4,6}$ and P_2 in equatorial conformations are similar in their general shapes, and display inflection points at the same pH* values.^{1}H NMR curves for H_2 and $H_{4,6}$ show unambiguously that this is not the case since the chemical shifts of the H_2 proton in an equatorial conformation are pH* dependent whereas those of $H_{4,6}$ are not. These observations have been interpreted by Horsley and Sternlicht[17] in terms of a 'through-space' electric-field effect, and a 'through-bond' inductive effect, which in general act oppositely, and which lead to the invariance of the chemical shifts of $H_{4,6}$ protons by cancellation of the two above-mentioned effects during the deprotonation processes. At pH* 9.7, H_2, P_2, $H_{1,3}$, $P_{1,3}$ are pH* dependent, but only H_2, $H_{1,3}$ and $P_{1,3}$ exhibit an inflection. The lack of inflection for P_2, which means that the pH* dependence is small for this nucleus, and the presence of an inflection for H_2, indicate that only the through-bond inductive effect occurs on H_2; the through-space effect is likely to be negligible. The small inflection at pH* 9.7 displayed by $H_{1,3}$ protons, similar to that of H_2 at the same pH* value, despite the large inflection displayed by $P_{1,3}$ phosphorus, is indicative of the presence of the two perturbations on $H_{1,3}$ methine protons. Since $H_{1,3}$ protons are upfield shifted, we can infer that the through-bond inductive effect predominates. We note that, whatever the pH* in the range 8.7–11.0, P_5 and H_5 are insensitive to the deprotonation processes. From the above considerations, it can be suggested that the inductive and through-space effects do not contribute to the chemical shifts of the H_5 proton.

In the pH* range 10.0–13.0 for phytic acid in axial conformations we observe that $H_{1,3}$ protons exhibit three small inflections at 10.7, 11.2 and 11.6 (like the phosphate groups), $H_{4,6}$ probably one at 10.7 and H_2 and H_5 present only one large inflection at pH* ≈ 11.6; there is a lack of chemical shift data for these protons at 10.0 < pH* < 11.3. These results suggest that (*i*) intramolecular interactions occur between all the phosphate groups; (*ii*) inductive and through-space effects contribute to the chemical shifts of $H_{4,6}$ and $H_{1,3}$ protons; the $H_{4,6}$ proton resonance does not shift very far in the pH* range 10.7–12.7, despite the deprotonation processes, which probably results in part from a quasi-cancellation of the two above-mentioned effects and (*iii*) at pH* 11.6, H_5 and H_2 are involved only in a through-bond effect. At pH* >11.8, all methine protons and all phosphorus are to some extent insensitive to pH*. One may then suggest that inductive and through-space effects do not contribute to the chemical shifts of protons.

CONCLUSION

We conclude that the stabilization of both axial and equatorial conformations of phytic acid cannot be explained only by minimization of electrostatic repulsions. The dependence of the conformations on multiple factors such as pH, P—O ··· H —O—P hydrogen bonds and C—H ··· O—P through-bond interactions, as in the case of hydrogen bond interactions of phosphate groups with water, supports this view. The occurence of through-space interactions in both axial equatorial conformations may explain that conformations, partly because of structural effects, either change or stabilize.

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