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^{31}P NUCLEAR MAGNETIC RESONANCE–pH TITRATIONS OF *myo*-INOSITOL HEXAPHOSPHATE

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ABSTRACT

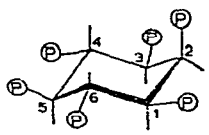
With the use of ^{31}P n.m.r. spectroscopy, the separate pK_a values of each of the six phosphoric monoester groups of *myo*-inositol hexaphosphate were determined. The range of hydrogen-ion concentrations covered extended from that required for the phosphonium salts to that for the full dodecyl anion, and the determinations were carried out in the presence of sodium and tetrabutylammonium cations. The pK_a for each phosphate grouping in the transition from the free acid forms of each group to the monoanion form of each group was determined to be: 1.1, C-2; 1.5, C-1 and C-3; 2.1, C-4 and C-6; and 1.7, C-5. In the mono- to di-anion transition, the pK_a values were: 6.85, C-2; 7.60, C-5; 5.70 and 12.0, C-1 and C-3; and 10.0, C-4 and C-6. These data and the appearance of the ^{31}P hexaphosphate n.m.r. multiplet are discussed in terms of conformations of *myo*-inositol hexaphosphate.

INTRODUCTION

In the realm of the biochemistry of cereal grains, the importance of phytic acid (*myo*-inositol hexaphosphate, a polyphosphorylated polyol sometimes incorrectly referred to as a polyphosphate) cannot be overemphasized. This substance, about which several reviews have been written[1–3], may account for as much as 80% of the total phosphorus of a grain, with as much as 90% of it being present in the germ[4]. Since 1903, when the existence of such material in seeds was first announced[5], considerable controversy has revolved about its precise nature, and, when it was shown that the substance actually comprises a family of polyphosphorylated inositols[1,6], the controversy was directed toward the precise chemical structure of the individual molecules, principally that of the ubiquitous *myo*-inositol hexaphosphate[7,8]. A number of different crystalline forms of sodium phytate are known that differ principally in their degree of hydration[9–11]. Undoubtedly, this pleomorphism contributed to the early confusion concerning the structure of phytates.

The primary structure was eventually firmly established through an excellent, early effort by R. J. Anderson[8], and then by Johnson and Tate[9], wherein, through the

use of advanced spectroscopic methods and condensing-agent chemistry to generate the model compounds needed, structure **1**,



Ⓟ = OPO_3H_2

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in the conformation indicated, was deduced for the form of the molecule in dilute, aqueous solution. Later, a single-crystal, X-ray analysis by Blank *et al.*[12] showed that, in the sodium salt hydrate containing 38 H_2O, the conformation was inverted, with the phosphate groups on C-1, C-3, C-4, C-5, and C-6 axially oriented and that on C-2 equatorially attached. These authors[12] considered this crystal structure to be "a realistic model for phytate in the solution state since it is highly hydrated and reflects the ionic character of phytic acid predictable under physiological conditions", and this was certainly a reasonable assumption, although it does not take into account the change in free energy associated with crystallization. The structure proposed by Johnson and Tate[9], which resulted from the interpretation of nuclear magnetic resonance (n.m.r.) spectra of dilute, aqueous solutions, was thought to be "inconsistent" with the "actual conformation" of phytate[12].

On applying phosphorus-31 n.m.r. spectroscopy to the study of the properties of naturally occurring, phosphorus-containing molecules, we observed that the ^{31}P resonance multiplet of *myo*-inositol hexaphosphate obtained from several different extracts of corn germ varied considerably in position and appearance, suggesting that the form of this phytate in aqueous solution, in analogy to that in the solid state, may also vary, and be quite sensitive to the specific nature of the solvating medium, *i.e.*, the type of cations present, the pH, and the ionic strength[13].

That the n.m.r. data from solutions, and crystallographic data from well-formed crystals, appear to be at variance is not unusual, For example, in the family of phospholipid derivitives, crystallographic X-ray studies on 2-aminoethyl phosphate by Kraut[14], and on 2-aminoethyl (1-deoxy-L-glycerol-1-yl phosphate) by De Titta and Craven[15], showed that these molecules exist, in the crystal, in a more or less linear conformation. However, quantum-mechanical investigations by Pullman and Berthod[16] on these same molecules indicated "that the polar heads of the phospholipids show an *intrinsic* preference toward highly folded structures with strong intramolecular hydrogen bonds". "Their existence in the open form in the crystals and possibly in water must be attributed to the effect of the environmental forces". Our ^{31}P n.m.r. studies on dilute solutions of these molecules support the interpretations derived from the quantum-mechanical investigations.

In this report, ^{31}P n.m.r. data obtained for a pure sample of *myo*-inositol hexaphosphate are presented, and the data as influenced by cations, pH, and ionic strength are discussed.

MATERIALS AND METHODS

Crystalline sodium$_{12}$ phytate (Sigma 69B-1500) was converted into the tetrabutylammonium salt by passing an appropriate solution through a column of Dowex-50 (H^+) ion-exchange resin, and immediately titrating the acid in the effluent with tetrabutylammonium hydroxide to the desired[17] pH. (In dealing with ^{31}P n.m.r. spectra of phosphates, it has been determined[18] that the tetrabutylammonium cation has the best overall solubility characteristics, and most closely approximates ideal cation behavior). The free acid and the phosphonium perchlorate[19] were also prepared, through the use of ion exchange[17], and dissolution in the appropriate aqueous perchloric acid solvents; the concentration of phytic acid employed was 10 mmolar. The samples so prepared showed no detectable impurities in the ^{31}P n.m.r. spectrum, even after prolonged signal-averaging to lessen the level of the background noise. Impurities not containing phosphorus constituted less than 5%, as indicated by wet-chemical[9,13] and chromatographic[4] procedures. Other reagents employed were commercial preparations.

The pK_a values reported were estimated in the usual way from plateaus and inflection points observed in the ^{31}P titration plots; the pH electrode used was a Sargent/Jena combination (glass) electrode, model S-30070-10, with a pH range of 0–14.

The n.m.r. spectrometer used was a Bruker HFX-5 instrument capable of heteronuclear (^2H and ^{19}F) field–frequency stabilization and operating at 36.43 MHz for ^{31}P, employing facilities for all modes of heteronuclear proton-decoupling, and possessing a Fourier-transform (16,384 data points) capability[13,20,21]. Except where otherwise indicated, measurements were conducted in the presence of a background of the appropriate cation chloride, and, because of the high purity of the sample, it was not necessary to employ (ethylenedinitrilo)tetraacetate as a scavenger for polyvalent ions[22]. Peak positions could be determined with a precision of ± 0.1 Hz in almost all cases, and pK_a values to a precision of ± 0.03 pH unit. As is customary in ^{31}P n.m.r. spectroscopy, the zero p.p.m. point corresponds to the resonance position of 85% orthophosphoric acid[19,23], and positive chemical-shifts are associated with increasing magnetic field strength[23]. The temperature of the solutions studied was 28°.

RESULTS AND INTERPRETATIONS

Free acid, and ammonium and phosphonium salts. — ^{31}P n.m.r. spectra of the ^{31}P-phytic acid multiplet are shown in Fig. 1 as it appears at hydrogen-ion concentrations corresponding to the four different ionic species of the six orthophosphoric monoester groupings in this molecule, namely, the dianion, the monoanion, the free

acid, and the phosphonium forms. Also shown in Fig. 1 is the proton-coupled spectrum of the multiplet obtained at pH 4.22. The entire, phytic acid multiplet is found in the orthophosphate region of the spectrum, at ~0 p.p.m., as expected for orthophosphoric monoesters[23], and each signal exhibits the characteristic POCH coupling (~9 Hz) to the single methine proton on the carbon atom bearing each phosphate group when proton-coupled spectra are recorded (see Fig. 1, coupled monoanion).

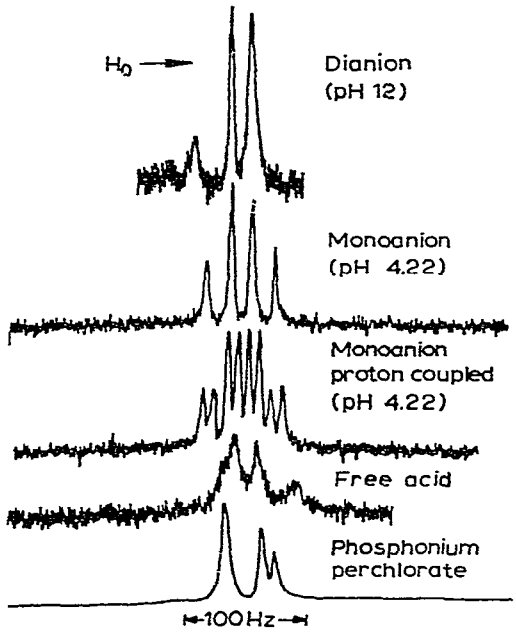


Fig. 1. ^{31}P n.m.r. spectra of the phytate (*myo*-inositol hexaphosphate) multiplet recorded at hydrogen ion concentrations corresponding to the four general ionic forms of the phosphoric monoesters (aqueous solutions, 10mM in phytate, with tetrabutylammonium, hydronium, or perchlorate counter-ions). [The spectra were obtained by signal-averaging and Fourier-transform, ^{31}P spectroscopic techniques by using 10-mm, spinning-sample tubes and internal, ^{2}H (10% HOD solvent) stabilization; all but the center spectrum are ^{1}H, broad-band spectra decoupled to suppress all ^{1}H couplings to the methine protons. Total sweep-width, 1.000 kHz (500 μsec/data point for 16,384 points); cycling time, 9 sec; temp., 28°. From 500 to 3,000 accumulations were needed for each spectrum. The multiplets all occur in the orthophosphate region of the ^{31}P n.m.r. spectrum; in the Figure, they are *not* aligned with respect to chemical shift.]

In the pH range from ~4 to ~10, at 28° and in the presence of the quaternary ammonium counter-cation, *myo*-inositol hexaphosphate gives rise to four proton-decoupled, ^{31}P resonance signals in the ratios of 1:2:2:1, the precise chemical shifts of which depend, to a first approximation, on the value of the pH*. Referring to

*Precise superposition of 2 or more resonances may occur at specific pH values in this range; see Fig. 2. However, the duplicity of the resultant resonance is readily seen from an examination of the ^{1}H-coupled spectra, where the different POCH coupling-constants can be observed.

formula **1**, which appears to be appropriate for this multiplet structure at pH 4.22, and reasonable with respect to the placement of the phosphate groups in relation to the ring, the assignments are as follows (see Fig. 1, decoupled monoanion). The most downfield signal arises from the single, axially attached phosphate group on C-2; following this, the high-field singlet of equivalent area arises from the unique, equatorially attached phosphate group on C-5, the second *meso* position. These assignments are consistent with respectively deshielded and shielded, axial and equatorial phosphate groups, and with previous ^{31}P assignments[9] for this molecule and ^{1}H assignments for the corresponding hexaacetate[24].

From considerations of proximity, it may be argued that the downfield partner of the two, prominent, inner signals arises from the (equivalent) phosphate groups on C-1 and C-3. These phosphate groups, each of which is neighbored by one axial and one equatorial phosphate group (respectively *cis* and *trans*), would be more exposed to the medium, and, hence, more deshielded than those on C-4 and C-6, each of which is neighbored by two equatorial phosphate groups (both in *trans* relationship).

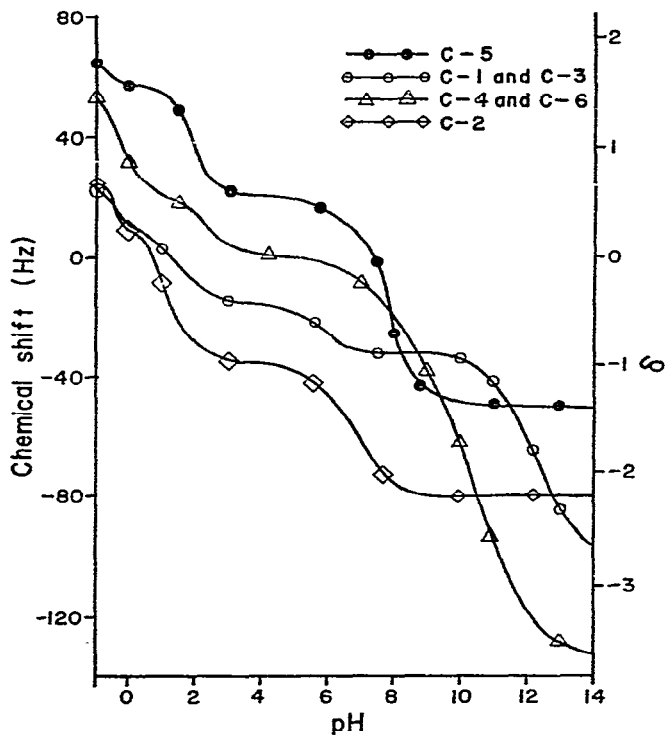


Fig. 2. ^{31}P n.m.r. titration curves of *myo*-inositol hexaphosphate (refer to formula **1**). [To obtain the data for all but the extreme values, appropriate volumes of an acid and alkaline solution, each prepared from the same, free acid solution, and both at the same concentration of phosphate, were mixed to obtain the samples for pH and ^{31}P n.m.r. analysis; on the sample to be analyzed, the pH was determined before and after the n.m.r. determination. For the sake of clarity, some data points are not presented in the Figure.]

Additional support for these assignments comes from variations in shift with change in pH, to be presented.

The line-widths of the separate signals were also observed to undergo considerable change with change in pH or counter-cation. Furthermore, line-width differences were usually observed within a multiplet obtained under any given set of conditions (see Fig. 1, pH 4.22 and 12). Preliminary T_1 relaxation-time and Overhauser enhancement studies with pH and counter-cation indicated that these differences arise from the changing T_1 and T_2 relaxation-times of the individual phosphate groups. The relaxation time–pH profile is, however, extremely complicated.

Fig. 2 presents the chemical shift of each signal as a function of pH (tetrabutylammonium counter-cation). These data are similar to those from the usual types of titration curve, and can be interpreted in terms of the pK_a values of the ionizing groups[25,26]. The transition from the monoanion to the dianion (pH >4) of the phosphate group on C-2 becomes increasingly more negative with increased ionization; the total range covers a little over 1 p.p.m. (46 Hz; pK_a 6.85). The phosphate group on C-5 undergoes a similar transition; the total range covered is 71 Hz, and the pK_a is 7.60.

The signal assigned to the phosphate groups on C-1 and C-3 shows two transitions in the range of pH values greater than 4, a small one (range, 17 Hz) exhibiting a pK_a of 5.70, and a more prominent transition (range, ~70 Hz) in strongly basic media having a pK_a of ~12. The change in chemical shift over the pH range from 4 to 12 is ~90 Hz. The signal assigned to the phosphate groups on C-4 and C-6 shows only a single, pronounced transition covering a range of 156 Hz and showing a pK_a value of 10.0.

Table I presents chemical-shift and coupling-constant data exhibited by multiplets of *myo*-inositol hexaphosphate corresponding to the various ionic species (equivalence points) which can exist for the separate orthophosphoric monoesters (see also, Figs. 1 and 2). Chemical-shift changes are again negative on going from the free acid form to the monoanionic species. The magnitude of the change is less, however, and is smallest for the prominent signals assigned to the phosphate groups on C-1 and C-3, and C-4 and C-6, and largest for the two *meso* positions, C-2 and C-5. Protonation of the phosphate groups to afford the corresponding phosphonium perchlorates, $R\text{–}O\text{–}P(OH)_3^+ClO_4^-$, results in the smallest chemical-shift changes observed with protonation.

In the transition from the free acid to the monoanion, exchange broadening of the phosphorus signals is observed. At a pH of 1.5, which corresponds to the approximate pK_a values of the various groups, exchange broadening is pronounced, and it changes the entire multiplet to a single, broad signal that is several hundred Hz in width. Nevertheless, on both sides of this value, data can be obtained, and it is possible to estimate the magnitudes of the various pK_a values to within ± 0.2 of a pK unit: C-2, 1.1; C-1 and C-3, 1.5; C-4 and C-6, 2.1; and C-5, 1.7.

Very little effect of protonation on the respective coupling-constants was observed (see Table I). The largest values were obtained from the signal of the phos-

TABLE I

^{31}P N.M.R. CHEMICAL-SHIFTS AND COUPLING-CONSTANTS EXHIBITED BY THE FOUR GENERAL IONIC FORMS OF *myo*-INOSITOL HEXAPHOSPHATE IN AQUEOUS SOLUTIONS WITH TETRABUTYLAMMONIUM, HYDRONIUM, AND PERCHLORATE COUNTER-IONS

Ionic form[a]	*Carbon atom numbers*[b]	*pH*	*Shift*		J^c (*Hz*)
			Hz[d]	*p.p.m.*	
Dianion	2	9.0	−80.1	−2.20	9.2
	1 and 3	>12	−102.0	−2.80	8.9
	4 and 6	>12	−132.0	−3.62	7.8
	5	10.0	−49.4	−1.36	10.4
Monoanion	2	3.8	−35.1	−0.96	9.2
	1 and 3	3.8	−14.4	−0.40	8.6
	4 and 6	3.8	4.3	0.12	8.6
	5	3.8	21.2	0.58	9.8
Free acid	2	0.0	10.0	0.27	—[e]
	1 and 3	1.0	3.1	0.09	—
	4 and 6	1.0	20.5	0.56	—
	5	0.0	57.5	1.58	—
Phosphonium cation	2	−1	24.5	0.67	7.0
	1 and 3	−1	22.7	0.62	6.3
	4 and 6	−1	53.5	1.47	8.3
	5	−1	64.5	1.77	11.4

[a]See Fig. 1 for the ^{1}H decoupled spectrum; refers to the ionic form of an individual ester grouping. [b]Refer to formula **1**. [c]Coupling constant. [d]^{31}P field equivalent to 36.43 MHz. [e]Signals were too broad for an accurate determination of coupling constant.

phate group on C-5, and the smallest values arose from the phosphonium form of the phosphate groups on C-1 and C-3. No obvious relationships between shift or pH, or both, and the coupling constants could be discerned, indicating little distortion in the various bond geometries.

The effect of ionic strength on the shifts of the hexaphosphate resonances is shown in Fig. 2. For all four cases, the shift change is linear with increasing salt concentration, but not necessarily in the same direction or to the same extent. Thus, the signal assigned to the phosphate groups on C-1 and C-3 exhibits a positive slope with increasing concentration of salt, whereas the remaining signals show a negative slope. Moreover, the signal assigned to the phosphate group on C-2 undergoes a more pronounced negative shift than that from the phosphate groups on either C-4 and C-6, or C-5, which show almost parallel behavior at pH 6.5.

At other pH values, the slopes exhibited by the phosphorus resonances in these plots of shift *versus* salt concentration are different; however, in each, change in shift is linear with salt concentration. Spot checks on the pH profiles at different ionic strengths showed that the relative differences between the shifts and the coupling constants remain the same, indicating that the changes in shift values with changes in ionic strength are not the result of alterations, induced by ionic strength, in the

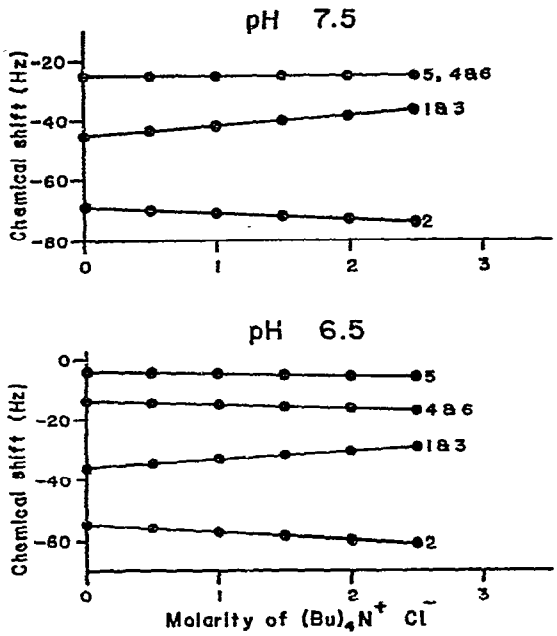


Fig. 3. The effect of added salt (tetrabutylammonium chloride) on the chemical shifts of *myo*-inositol hexaphosphate (refer to formula 1).

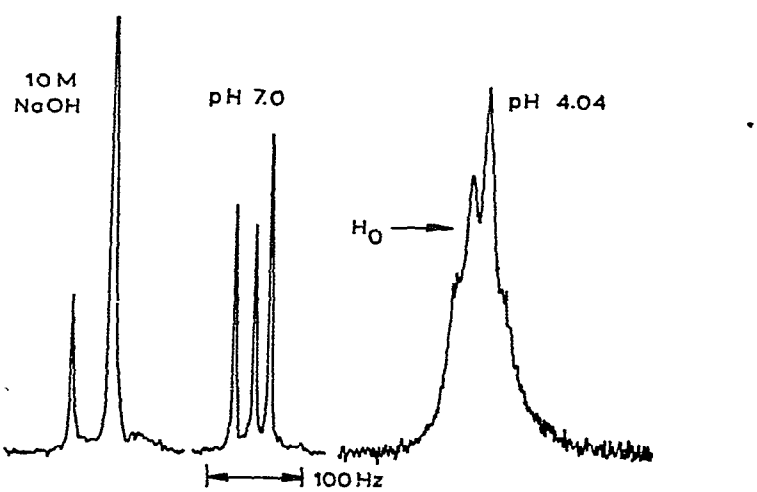


Fig. 4. ^{31}P n.m.r. spectra of *myo*-inositol hexaphosphate with sodium as the counter-cation. [The experimental parameters are similar to those given in the caption to Fig. 1.]

relative acidity of the phosphate groups. Furthermore, these changes are linear within the precision of measurement, whereas pH-induced shift-changes are sigmoidal. In addition, experiments on ionic strength conducted with tetrabutylammonium bromide, iodide, sulfate, and perchlorate (as well as the chloride) showed that these changes are also independent of the anion associated with the salt added.

Sodium salts. — When sodium is the counter-cation, the appearance of the ^{31}P multiplet of *myo*-inositol hexaphosphate is changed considerably, especially in the physiological range of pH. Fig. 4 shows the ^{31}P n.m.r. spectra of sodium phytates recorded at three representative values of pH. At pH 4.04, or at any lower value, the four-line pattern shown by the ammonium salts is also observed for the sodium salts. Like those of most simple orthophosphoric monoesters, the shifts in the presence of sodium ion are to fields lower than those in the presence of ammonium ions. In addition, for the multiplet of *myo*-inositol (sodium hexaphosphate), the individual signals are more tightly grouped, showing smaller differences in their respective, chemical-shift values. The assignments made for the ammonium compound hold also for the sodium salt at pH values equivalent to, or lower than, that corresponding to monoprotonated phosphoric monoesters.

At pH 7.0, the sodium hexaphosphate gives rise to the ^{31}P multiplet shown in the middle of Fig. 4. The multiplet consists of three signals of equal area. No other resonances were observed in the ^{31}P n.m.r. spectrum.

In 10M sodium hydroxide, the multiplet of *myo*-inositol hexaphosphate is lessened to two signals having relative areas of 1:5. Under these conditions, the minor resonance can be assigned to the single, axially attached phosphate group on C-2 in **1**. The major resonance must arise from the remaining five, equatorially attached, phosphate groups. The single, sharp resonance indicates that these must also experience equivalent local magnetic fields.

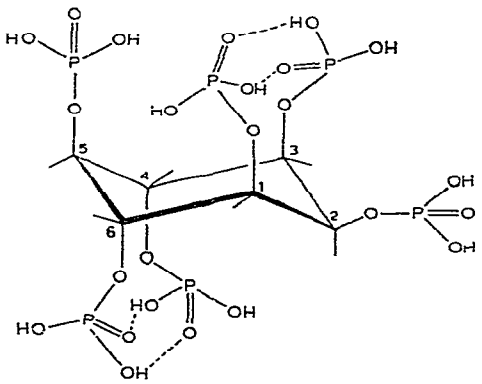
DISCUSSION

Phytic acid provides an excellent model for the study of the ^{31}P n.m.r. behavior of interacting phosphoric monoester groupings. Each monoester of the hexaphosphate is a functionally equivalent chemical group, with the groupings differing only in their spatial relationships to one another, and to the plane of the carbon skeleton.

Ionization constants and conformation. — With respect to broad general features, the pK_a values that we have found for the ionizable groups agree with earlier interpretations based on potentiometric-titration data[27]. The differences between our data and those obtained earlier can be attributed, in part, to our use of the tetrabutylammonium instead of the sodium ion in the determinations of pK_a. Furthermore, as each phosphate group is observed separately in ^{31}P n.m.r. spectra, it is possible to obtain more-precise values for the individual pK_a values. We found three very weak acids (pK_a 10 and higher); two were in the usual, weak-acid range for phosphoric monoesters (pK_a 6.8–7.6), and one was a stronger acid having a pK_a value of 5.70. Earlier data[27] were interpreted as indicating four functions having a pK_a ~9.7, and

two at lower values (pK_a 6.3). The six strong-acid pH_a values are clustered about a value of ~1.5 (1.84 according to ref. 27).

The fully protonated structure **2** was formulated by Courtois and co-workers[27] in their conclusions concerning the structure of *myo*-inositol hexaphosphate based on potentiometric data. The protons involved in the hydrogen bonds correspond to the



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four weak acids that they observed having pK_a values of 9.7. The structure is unusual, in that five of the phosphate groups are situated in axial positions on the six-carbon ring. It had been postulated[2] that such a structure would "no doubt (be) associated with a very high energy content". The conformation shown in structure **2** is the one that has been determined[12] in the sodium phytate·38 H_2O crystal.

The n.m.r.-spectral data, especially those of the sodium salts (see Fig. 4), suggest, however, the possibility that the conformation corresponding to structure **2** is not the only form in which phytate may exist in dilute, aqueous solution. It seems reasonable to suggest that the conformation corresponding to structure **1** exists in concentrated sodium hydroxide solutions, and that a conformation containing three pairs of equivalently shielded phosphate groups exists in neutral solutions containing sodium ions. Such a structure may account for the formation of the stable octadecasodium and trihydrate salts that originally led to Neuberg and Popowsky's[7] formulation for phytate, now shown to be incorrect. Moreover, the suggestion that the conformation of phytate may change to a boat as the ionic strength increases in the tetrabutylammonium system is not entirely inconceivable. Such a conformation is considerably more compact and, hence, would be favored over the chair conformations as the solvent is depleted. Also, such a structure is not inconsistent with the ^{31}P n.m.r. data. Other conformations may be proposed, but no conformation can at present be established with certainty, as n.m.r. proofs for any proposed structure must rest upon subtle and often delicate spectroscopic arguments.

The important point (see also Brown and Tate[28], where a similar conclusion is drawn) is that conformations of phytic acid other than those corresponding to formula **2** may exist in solution under certain conditions, and these conformations may,

perhaps, be locked into crystal structures through the judicious choice of counter-cation or pH, or both, and, hence, allow verification through X-ray crystallography.

Long- and short-range shielding-effects. — The chemical shifts of all six phytate phosphate groups were found to be dependent on both the ionic strength and the hydrogen-ion concentration of the solvent. Among these signals, the resonance assigned to the phosphate group on C-5 was least dependent on the solvent, undergoing 0.03–0.04 p.p.m. deshielding with a 2.5-fold increase in ionic strength, and a 0.003 p.p.m. deshielding with an equivalent, 2.5-fold increase in hydrogen-ion concentration. The signal assigned to the phosphate group on C-2 underwent 1.4 to 1.6 p.p.m. deshielding with the same 2.5-fold increase in ionic strength, and 0.006 p.p.m. deshielding with the equivalent increase in hydrogen-ion concentration. The results from the signals assigned to phosphate pairs on C-1 and C-3, and on C-4 and C-6, show opposite responses to changes in ionic strength and pH. With the same, 2.5-fold changes in the solvent, the signal assigned to the groups on C-1 and C-3 is most affected by ionic strength (0.22 to 0.19 p.p.m. increase in shielding), and least affected by pH (0.007 p.p.m. deshielding), whereas the signal assigned to the groups on C-4 and C-6 is least affected by ionic strength (0.03 to 0.09 p.p.m. deshielding) and most affected by pH (0.01 p.p.m. deshielding).

Solvent-induced changes in chemical shift may be explained by two general approaches. In the first, which is generally called "long-range" nuclear shielding, distant atoms or groups exhibiting spin behavior act together to modify the magnetic field applied as experienced by the nucleus under observation[29]. The other[23,30] considers the effect of the "unbalanced *p* and *d* electrons" (ref. 31) of the nucleus under observation that is brought about by changes induced in its electronic interaction with neighboring atoms (the electronic structures of which are, in turn, affected by local changes in their environment). Although either of these theoretical approaches may be used independently to explain either an increase or decrease in chemical shift due to solvent changes (such as the incorporation of a salt into a solvent, or change in the hydrogen-ion concentration), they are basically different (long-range *versus* short-range shielding), and real situations may involve both of them.

For the six phosphate groups of phytic acid, there is some information that may be used to distinguish which, if either, of these two alternatives predominates. Were long-range shielding dominant, the various phosphate groups would show the same slope with increase in salt concentration (see Fig. 3), and almost equivalent pK_a values for each successive degree of ionization of the phosphoric monoester groupings (see Fig. 2; here, the "associated" proton is considered as being *in* the solvent), but this is not the case. Furthermore, the shift changes induced by ionic strength are independent of the anion of the added salt, and this could not have been anticipated. Thus, the data strongly indicate that the chemical-shift changes observed must be attributable to short-range interactions between the cations or the water solvent, or both, and the orthophosphate anion under n.m.r. observation. This kind of situation is probably best handled by a treatment such as that which has previously been applied[32] to the interpretation of the change in ^{31}P chemical shifts of (*a*) some

oxyacids of phosphorus with degree of neutralization, or (*b*) a series of ethyl phosphates with changing ionic strength[33].

Approximate quantum-chemical calculations[30] have shown that the change in the chemical shift ($\Delta\delta$) for such compounds may be treated by the relationship[32]

$$\Delta\delta = 180\Delta\chi_0 - 147\Delta\eta_\pi - A\Delta\theta, \qquad (1)$$

where $\Delta\chi_0$ is the change in the effective electronegativity of the PO_4 oxygen atoms caused by the change in the solvent, $\Delta\eta_\pi$ is the concomitant change in the phosphorus d_π-orbital occupation due to variation in the π character of the P–O bonds thus induced, and $\Delta\theta$ is any change in the O–P–O bond-angle caused by the alteration of the solution. As A is a small number lying in the range of 0–0.3, the expected small change in bond angles can surely be neglected. From this equation, it is clear that an *increase* in ^{31}P chemical-shift upon addition of a salt corresponds to *more σ-electron withdrawal* by the oxygen atoms from the phosphorus, or *less* $p_\pi \rightarrow d_\pi$ donating by the oxygen atoms to the phosphorus atom, or both. Therefore, we conclude from eq. *1* that the signals assigned to the phosphate groups on C-2, C-5, and C-4 and C-6, which are "deshielded" with increase in salt (see Fig. 3), receive electrons from the oxygen atoms of the PO_4 groups. Such would occur were there less interaction of the PO_4 oxygen atoms with the solvent-water molecules and more self-interaction within each phytate residue. This interpretation is consistent with depletion of the solvating water with increase in salt concentration. The opposite interpretation would hold for the signal assigned to the phosphate groups on C-1 and C-3, *i.e.*, it appears that these phosphate groups are projected into the solvating medium.

In all six instances (see Fig. 2), association of a proton results in decreased electron-donating power for the PO_4 oxygen atoms and is consistent with site-binding of the associated proton with a specific PO_4 group. Site-binding of the protons is also apparent from the differing pK_a values of the various phosphate groups of phytate.

Apparent additive shifts for interacting phosphate groups. — It should be noted that the magnitude of the difference in chemical shift observed between the fully ionic form of a phytic monoester, $ROPO_3^{2-}$, and its corresponding monoprotonated species, $ROPO_2(OH)^-$, appears to be an additive function of the number of phosphate groups contributing to the resonance. Thus, on ionization in the pH range 4 to >12, which covers this transition (see Fig. 2), the signal assigned to the phosphate groups on C-1 and C-3 undergoes a 90-Hz change in chemical shift which, were the contributions of each phosphate group additive, amounts to 45 Hz per phosphate group. The corresponding change in chemical shift observed for the interstitial phosphate on C-2 is 46 Hz. Similarly, the signal assigned to the phosphate groups on C-4 and C-6 undergoes a change of 156 Hz (68 Hz per P atom), whereas the interstitial C-5 signal changes by 71 Hz. Although there is no theoretical reason for assuming that the shift changes among interacting phosphate groups should, upon protonation, be additive, the correlation among the values is, nevertheless, striking.

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