A Novel Technique for the Pre-Concentration and Extraction of Inositol Hexakisphosphate from Soil Extracts with Determination by Phosphorus-31 Nuclear Magnetic Resonance

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ABSTRACT

Inositol hexakisphosphate (IP$_6$) is often the dominant form of soil organic phosphorus (P), but is rarely investigated because of the analytical difficulties encountered in its extraction, separation, and detection in environmental samples. In particular, recent advances in the study of soil organic P with ^{31}P nuclear magnetic resonance (NMR) have been of limited use for the study of IP$_6$, because the technique does not discriminate between IP$_6$ and other forms of P. This was addressed by developing a novel analytical procedure using the retentive properties of gel-filtration gels for IP$_6$, which allows the combined selective extraction and pre-concentration of IP$_6$ from soil extracts with determination by ^{31}P NMR. While the technique is still in the developmental stage, the results demonstrate that the gel does not interfere with ^{31}P NMR analysis and retains IP$_6$ to concentrations well above those required to give clear spectral signals. The technique has considerable potential for application to the study of IP$_6$ in soil extracts and water samples and, with development, could help to answer fundamental questions regarding the dynamics of organic P in the environment.

THE MOST ABUNDANT CLASS of organic phosphorus (P) compounds in the environment is the inositol phosphates, a family of six congeners of hexahydroxy cyclohexane (inositol) that exist as inositol in various states of phosphorylation (bound to between 1 and 6 phosphate ions) (Fig. 1). Nine stereoisomers of inositol phosphates exist; the *myo* stereoisomer is by far the most common in nature, although *neo-*, *scyllo-*, and *chiro*-inositol phosphates have been reported in terrestrial and aquatic environments (Cosgrove, 1980). The dominant form of inositol phosphate in the environment is *myo*-inositol hexakisphosphate (IP$_6$), which constitutes the major organic P compound in soils and aquatic sediments (Harrison, 1987; Suzumura and Kamatani, 1995). Despite its abundance, it remains poorly understood and little reliable information exists on the sources, pools, and dynamics of IP$_6$ in the environment (Turner et al., 2002). The role of IP$_6$ in supplying P to plants and algae is largely unknown and even its origins remain unclear in many cases (L'Annunziata, 1975). Research into IP$_6$ has been limited by the lack of suitable analytical techniques for its determination in environmental samples, the main problems being poor recoveries of IP$_6$ from soils by conventional extractants and from anion exchange columns during sample cleanup and separation (Anderson, 1964; Martin, 1970; Irving

and Cosgrove, 1981). Furthermore, the concentrations of IP$_6$ in environmental samples are too low for most analytical techniques, although new pre-concentration procedures have recently been developed that can address this (Nanny et al., 1995; Espinosa et al., 1999). These problems must be overcome before significant advances can be made in understanding IP$_6$ dynamics (Turner et al., 2002).

This work describes a new approach to the use of ^{31}P NMR for the determination of IP$_6$ in soil extracts and water samples. Nuclear magnetic resonance is a powerful tool for the investigation of P forms in soil extracts (Condron et al., 1997), but it is limited for IP$_6$, because of an inability to separate IP$_6$ from orthophosphate and other phosphomonoester compounds. This is because phosphomonoester signals (including IP$_6$) appear as a single envelope and frequently overlap with the orthophosphate signal, making quantification difficult (e.g., Hawkes et al., 1984; Condron et al., 1990). Further problems are encountered with interfering paramagnetics (such as Fe^{3+} and Mn^{2+}), which cause line broadening and can require removal from the sample by chelating resins. Other problems are the low concentrations of organic P in soil extracts (relative to those required for ^{31}P NMR), which means some form of pre-concentration is necessary. However, ^{31}P NMR provides a simple method of determination compared to the time-consuming and complex separation and detection procedures used in previous studies of IP$_6$ (e.g., Anderson, 1964; Martin, 1970; Irving and Cosgrove, 1981).

Inositol hexakisphosphate can be retained by gels used for molecular size separations such as Sephadex (Martin, 1970; McKelvie et al., 1993), although adsorption varies depending on solution parameters. For example, McKelvie et al. (1993) showed that optimum (but not complete) adsorption of IP$_6$ to Sephadex G-25 gel occurred at ionic strengths >0.05 M and pH between 7.5 and 10, although Condron and Goh (1989) showed that there was negligible retention of soil organic P in NaOH extracts onto Sephadex G-100 gel columns. The adsorptive properties of these gels has presented a major problem for investigation of organic P in soil extracts and water samples (Martin, 1970; McKelvie et al., 1993), but provides a potential means of separation. We investigated the potential use of the retentive property of Sephadex G-25 gel for the selective extraction of IP$_6$ from a solution, followed by analysis with ^{31}P NMR. The proposed technique overcomes several of the major problems of determining IP$_6$ in soil extracts with ^{31}P NMR, namely the selectivity of extraction, pre-concen-

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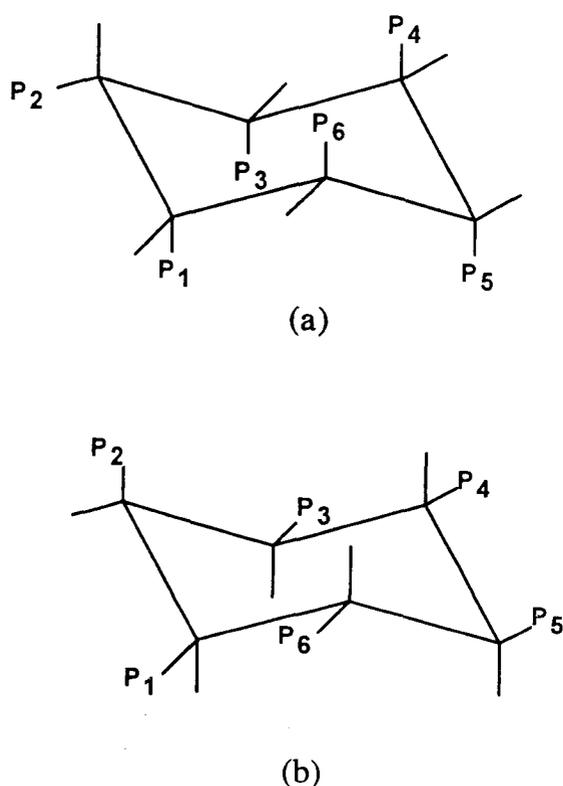
Fig. 1. (*a*) Axial form of phytic acid (pH 5–12) and (*b*) equatorial form of phytic acid (pH < 5 and pH > 12) (Martin and Evans, 1986). $P_i = -OPO_3^{2-}$, i = 1,2...6.

tration, and preclusion of interference by other P compounds.

METHODS

Principle of the Method

Sephadex is manufactured by cross-linking dextran with epichlorohindrin; varying the degree of cross-linkage creates gels with different porosities. Sephadex gel has been shown to retain IP_6, while excluding other organic P forms and orthophosphate (McKelvie et al., 1993). By using this property, IP_6 can be removed from soil extracts in the presence of the gel, while impurities and other P compounds are removed by centrifugation, decantation, and washing prior to analysis with ^{31}P NMR.

Sample Preparation

One-gram Sephadex G-25 (fine) gel powder (giving approximately 7 mL of gel) was mixed and, therefore, hydrated in 10 mL of ultrapure water containing 1 mg P as phytic acid (magnesium-potassium *myo*-IP_6). A blank sample (no P) was included. The gel was allowed to swell and equilibrate by shaking end-over-end in 25-mL centrifuge tubes overnight at approximately 20°C. The mixtures were centrifuged for 5 min at low speed (<500 × g) to settle the gel and the supernatant was decanted and retained for total P analysis by sulfuric acid-persulfate digestion (Rowland and Haygarth, 1997). The remaining gels were then washed in ultrapure water and re-centrifuged. The washed gels were resuspended in 5 mL ultrapure water to form a slurry, which was poured carefully into 10-mL NMR tubes. Aliquots of the initial supernatant solutions were also poured into NMR tubes, as was a sample of

Table 1. Recoveries of model phosphorus (P) compounds (%) from solutions containing 1 mg P after mixing with 1 mL Sephadex gel overnight at 20°C. Values are means of triplicate samples ± standard error.

P compound	Recovery
	%
β-glycerophosphate (disodium salt)	97 ± 0.9
DNA (degraded free acid from herring sperm)	96 ± 0.8
Glucose-1-phosphate (dipotassium salt)	95 ± 0.8
Potassium dihydrogen orthophosphate	96 ± 1.6
Tetrasodium pyrophosphate	97 ± 1.4

the phytic acid stock solution (100 mg P L^{-1}). These samples were run for approximately 100 scans.

To test the selectivity of Sephadex gel for IP_6, the procedure was repeated for a range of other organic and inorganic P compounds (Table 1). Samples were prepared as described above and the supernatant solutions were analyzed for total P. Recoveries are expressed as the percent recovery from solutions containing 1 mg P and are reported as means plus or minus the standard error of triplicate samples.

To test whether Sephadex would retain IP_6 to concentrations at which acceptable signal to noise ratios could be achieved, samples were prepared with 1 mL of gel (0.143 g powder) in 50 mL centrifuge tubes. For this experiment, Sephadex-IP_6 mixtures were prepared according to the conditions for optimal retention of phytic acid by the gel (McKelvie et al., 1993). Forty milliliters of IP_6 solutions containing 40 and 20 mg P as IP_6 were mixed with Sephadex powder and 1 mL of 1.0 M Tris-HCl buffer, pH 8, containing 2 M NaCl (24 mM Tris, 49 mM NaCl final concentrations) and shaken overnight as described previously. These samples were run for approximately 700 scans.

Soil Extract

A soil extract was prepared for analysis by the Sephadex gel method. Two grams of soil (fine sandy clay loam, USDA Haplustult; total carbon 4.7%, pH in water 5.0, sand 38%, silt 49%, clay 13%, total P 685 mg kg^{-1}) was shaken for 15 h overnight in 40 mL of 1 M NaOH, at a 1 to 20 soil to solution ratio. This was centrifuged for 1 h at 10 000 × g, filtered through an Advantec No. 2 filter paper (Advantec Toyo Kaisha Ltd., Tokyo), and 10 mL of solution was prepared for NMR analysis as for the IP_6 samples described previously.

Phosphorus-31 Nuclear Magnetic Resonance Analysis

The "gel-slurry" samples were analyzed with ^{31}P NMR, using a Bruker Advance DRX400 spectrometer (Bruker, Germany) (which uses a ^{31}P operating frequency of 162 MHz), and data were collected with broadband (waltz) proton decoupling. The relaxation delay was 2.5 s, acquisition time was 0.84 s, with an approximate 40 000-Hz sweep width, 64 K data points for acquisition and 131 K for processing. The samples were run unlocked (no D_2O). The number of scans was sufficient to obtain a recognizable signal. The 0 ppm position corresponded to the resonance of 85% orthophosphoric acid as the external reference and positive chemical shifts corresponded to increasing magnetic field strength (Costello et al., 1976).

RESULTS

No ^{31}P NMR signal was present for the blank sample (ultrapure water + Sephadex gel), indicating that the presence of Sephadex would not interfere in the ^{31}P
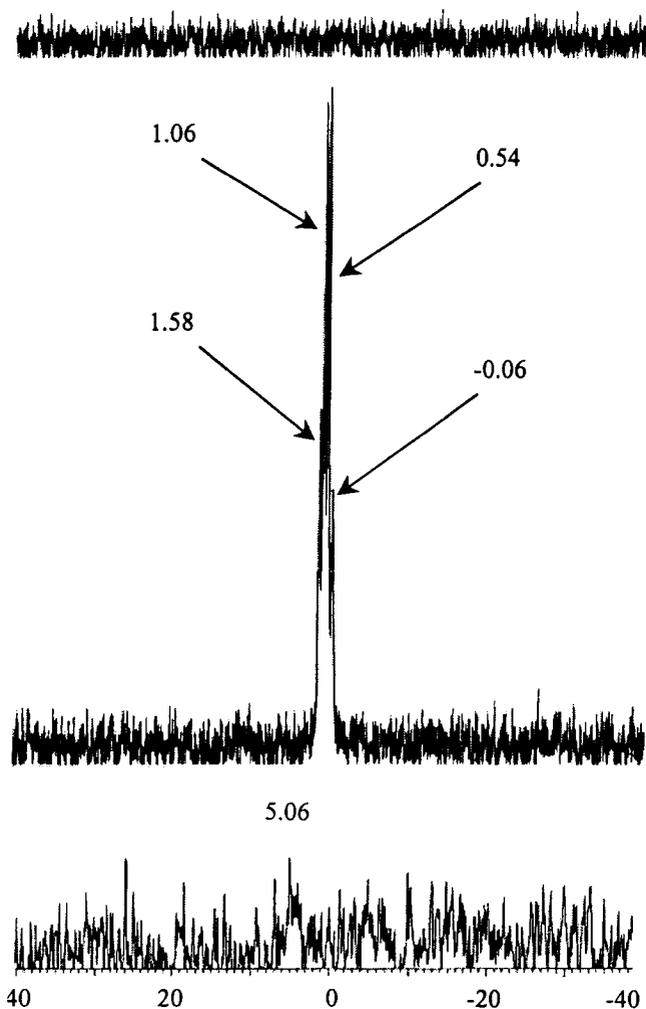
Fig. 2. Phosphorus-31 nuclear magnetic resonance (NMR) spectra of a blank sample containing Sephadex G-25 (fine) gel and ultrapure water (top), a sample containing 1 mL of Sephadex gel and 40 mg P as phytic acid (center), and a sample of Sephadex gel that had been mixed with 10 mL of 0.5 M NaOH soil extract (bottom).

NMR analysis (Fig. 2). The total P concentration in triplicate blank samples was 3.1 μg L^{-1} (SE = 1.76 μg L^{-1}) and was, therefore, below the limit of detection for total P in water (Rowland and Haygarth, 1997). The Sephadex + IP$_6$ sample showed a quadruplet signal in the range of +0.098 to +1.428 ppm, identical to the spectra of the IP$_6$ standard solution (spectra not shown). The supernatant of the Sephadex + IP$_6$ mixture showed no signal, indicating that the gel had completely retained IP$_6$ from solution, which was confirmed by analysis of the supernatant for total P by sulfuric acid-persulphate digestion. Furthermore, other model P compounds were not retained by the Sephadex gel, as indicated by less than 95% recoveries of orthophosphate, pyrophosphate, DNA, β-glycerophosphate, and glucose-1-phosphate (Table 1).

The 20- and 40-mg phytic acid-P samples gave quadruplet peaks in similar areas between −0.06 and +1.58 ppm, respectively (see 40 mg sample, Fig. 2). This indicated that the gel could retain IP$_6$ to concentrations well above those required to give clear spectral signals. The

peak heights of these two samples were not in proportion, indicating that the limit for IP$_6$ retention by the gel lies somewhere between the two concentrations (based on 1 mL Sephadex gel).

The soil extract sample showed a small signal at +5.063 ppm (Fig. 2), consistent with the position of IP$_6$ in alkaline soil extracts (Newman and Tate, 1980), but even after more than 32 000 scans this peak was not clearly distinguishable from the background noise, indicating that the IP$_6$ concentration was too low for effective analysis. Clearly, some form of pre-concentration would be required for application to real samples.

The results, therefore, showed that (i) the Sepahadex gel did not interfere with P determination by ^{31}P NMR, despite the 'solid' nature of the matrix; (ii) phytic acid was completely retained by the Sephadex gel to concentrations above those required to give clear spectral signals; (iii) orthophosphate and other P compounds were not retained by the Sepahadex gel; and (iv) soil extracts would need pre-concentration prior to analysis with ^{31}P NMR.

DISCUSSION AND FUTURE DEVELOPMENT OF THE METHOD

These preliminary results are promising, although substantial development is required. The study demonstrated that the basic principles of the method are sound, because Sephadex gel did not interfere with ^{31}P NMR analysis and successfully retained IP$_6$ from solution, while excluding other P compounds.

The quadruplet signal obtained for phytic acid has also been reported by other workers, although the chemical shift and number of peaks varies depending on the pH and the ionic strength of the sample, particularly the concentration of sodium (Costello et al., 1976). For example, at pH in the range of 4 to 10, phytic acid gives four resonance signals in the ratios of 1:2:2:1, which correspond to the positions of the orthophosphate ions on the inositol molecule.

Pre-concentration appears to be a fundamental prerequisite for this technique, but could be achieved relatively simply by preparing a small column containing 1 mL of Sephadex gel, through which a buffered stream of sample could be passed with a peristaltic pump. The gel would retain IP$_6$, with the remaining sample containing other P compounds passing to waste. Presumably, measurable concentrations of IP$_6$ from soil extracts could be obtained by passing sufficient volumes of extract through the gel column. The use of the common extracts for soil organic P, such as NaOH-EDTA (Cade-Menun and Preston, 1996), would require strong buffers to achieve the required pH for optimal retention of IP$_6$ by the gel. However, this pre-concentration procedure would allow the determination of IP$_6$ in waters given sufficient sample volume.

Quantification could be achieved by running a suitable range of IP$_6$ standards, or more simply by including an internal reference standard containing a known concentration of P in a form that would not interfere with the peak of IP$_6$ in a capillary tube inside the NMR tube.

For example, Hinedi et al. (1988) used phosphonate as an internal reference standard (chemical shift around +20 ppm). In this case, the P concentration would be calculated from the ratio of peak area of the sample with that of the internal standard. An additional benefit would be that quantification would not require a predetermined number of scans, but could be determined whatever the number of scans required to obtain a measurable signal.

Application of the technique to soil extracts might be further complicated by the retention by the gel of paramagnetics like Fe^{3+} and Mn^{2+}, which interfere with the determination of IP_6 by ^{31}P NMR (Condron et al., 1997), although these compounds are reportedly not the cause of the poor resolution of solid-state ^{31}P NMR signals of soils (Shand et al., 1999). A greater problem may be the nature of organic matter–IP_6 associations in soils, which could protect IP_6 from interacting with the gel. The prevalence of these associations in soils may necessitate the use of hypobromite oxidation prior to pre-concentration. This technique oxidizes all organic matter except inositol phosphates (Irving and Cosgrove, 1981; Nanny and Minear, 1997) and would free IP_6 to solution, from where it could be extracted, pre-concentrated and analyzed by the Sephadex-NMR procedure.

The technique has considerable potential for the quantification of IP_6 in soil extracts and a range of other environmental samples, including sediment extracts and waters, and might facilitate the determination of IP_6 as a routine analytical technique. Interestingly, there is the possibility of extending the technique to ^1H NMR for the identification (and possibly quantification) of the isomers of IP_6, which has been demonstrated in solutions (Suzamura and Kamatani, 1993). This would give complete identification of the IP_6 component.

An interesting application of the technique could be for the determination of 'labile' phosphomonoesters in soil extracts by ^{31}P NMR. Currently, ^{31}P NMR cannot distinguish individual phosphomonoesters, such as sugar phosphates, mononucleotides, and IP_6, because the spectral signals overlap. The IP_6 component dominates in most soils, yet other phosphomonoesters are probably more important in terms of short-term cycling and availability to plants. The Sephadex technique described here could be used to "clean up" soil extracts by removing IP_6 and allow other phosphomonoesters to be quantified with ^{31}P NMR.

CONCLUSIONS

The feasibility of using Sephadex gel for the selective extraction and pre-concentration of IP_6 from solutions and detection with ^{31}P NMR has been investigated. The technique is still at the developmental stage, but has considerable potential for quantifying IP_6 in soil extracts and water samples. This would contribute considerably to our understanding of IP_6 dynamics in the environment. Additional development is required to make the method quantitative and to investigate the potential problems involved in its application to soil extracts.

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Flow and Diffusion Measurements in Natural Porous Media Using Magnetic Resonance Imaging

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ABSTRACT

Flow and diffusion of water in natural porous media, quartz sand, and calcareous gravel were measured using a 1.5-T clinical magnetic resonance tomograph. The spatial resolution of the dynamic measurements was $1.32 \times 1.32 \times 5$ mm³, and the time between two cross-sectional measurements was approximately 10 s. The measured coefficients of molecular diffusion for water were in good agreement with theoretical data. Flow was measured without any tracer at velocities between 0.15 and 6.67 mm/s. The results, based on a calibration within one part of the column, were in good agreement with data obtained from a tracer experiment and from a numerical model. It was possible to measure the flow velocity in larger pores and preferential flow paths directly. The results of the flow measurements in smaller pores reflected the mean velocity within that volume element. In that case the obtained values were close to the average linear velocity. Since the time resolution is high a monitoring of flow processes is possible. The pore space was imaged with a spatial resolution of $0.5 \times 0.5 \times 0.5$ mm³. Here, the porosity of pores that are larger than 0.2 mm can be measured directly; for smaller pores a calibration is necessary.

THE FLOW VELOCITY in natural porous media is of high relevance to contaminant and colloidal transport in subsurface environments. The transport of dissolved contaminants is subject to sorption and desorption processes with a defined sorption kinetic (Wu and Gschwend, 1986; Song et al., 1994), and the flow velocity at the pore scale determines the extent of dynamic sorption processes. The spatial distribution of dissolved contaminants is again a function of the flow velocity on a pore scale (Corapcioglu et al., 1997). The different flow velocities and different flow paths are the reason for dispersion. In a three-phase system, consisting of fluid, colloids, and stationary matrix, the colloids may facilitate contaminant transport (McCarthy and Zachara, 1989). Apart from surface interactions, the transport of the colloids is depending on the pore space itself (pore-size distribution, connectivity) and on the flow velocity within the pores (Harvey and Garabedian, 1991; Rehmann et al., 1999). Preferential flow paths (e.g., worm-

holes, fractures, or sediments with a higher flow velocity) contribute to the transport behavior of dissolved and colloidally bound contaminants.

From the physical description of the transport processes it becomes quite clear that the average linear velocities, as obtained from, for example, tracer experiments, are an approximation. The linear distance between the injection and monitoring locations is always the shortest possible distance and does not account for the actual flow path, nor for the different flow velocities in larger and smaller pores, or for the parabolic flow profile in a pore. These factors are summarized as mechanical dispersion. Still, mechanical dispersion is calculated without knowledge of the length of the actual flow path. Thus, the flow velocities calculated from tracer experiments are always slower than the actual flow velocities at the pore scale. The differences become relevant, if a numerical model includes the sorption kinetic measured in laboratory experiments or derived from thermodynamic databases, or if filtration theory is applied for colloid transport phenomena. In those cases the knowledge of the flow velocity at the pore scale would be useful to scale laboratory experiments to real-world problems. There is also need for (near-) real-time measurements of flow and diffusion and the spatial evolution of the pore space to investigate, for example, the obstruction of pore spaces by colloids (Veerapaneni and Wiesner, 1997) or the development of preferential flow paths in the time domain.

Among the techniques for a spatial description of the pore space, thin sectioning and light microscopy are quite common (Vogel, 1997; Vogel and Roth, 1998). The use of X-ray computer tomography (CT) is reported for the characterization of the pore space of soils (Reinken et al., 1995) and glass beads (Jasti et al., 1993). In the context of oil exploration, CT was used to determine the pore space (Akin et al., 1996) or to characterize the swelling behavior of shales (Onaisi et al., 1993). Computer tomography has a good spatial resolution, down to 5 μm in high-resolution mode (Reinken et al., 1995). However, CT measurements of flow processes have several drawbacks. Primarily, CT images the sediment matrix, but the fluid itself yields almost no signal

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