Identification of scyllo-Inositol Phosphates in Soil by Solution Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

Benjamin L. Turner* and Alan E. Richardson

ABSTRACT

A large proportion of the organic P in soils can occur as scyllo-inositol phosphates. These compounds are rarely detected elsewhere in nature and remain poorly understood, partly because conventional procedures for their determination are lengthy and error-prone. We report a straightforward procedure for the determination of scyllo-inositol phosphates in soil extracts using solution 31P nuclear magnetic resonance (NMR) spectroscopy. Solution 31P NMR chemical shifts of a range of synthetic scyllo-inositol phosphate esters were determined in alkaline solutions. Of these, only the signal corresponding to scyllo-inositol hexakisphosphate is identified in soil NaOH–EDTA extracts, constituting between 6.5 and 9.8% of the NaOH–EDTA-extracted P. This signal has been previously assigned to choline phosphate, but we confirmed it to be an inositol phosphate using hypobromite oxidation, a procedure that destroys all organic matter except inositol phosphates. Lower order scyllo-inositol phosphate esters were not identified in the extracts studied here, and literature reports suggest that they probably occur in insufficient concentrations to be detected by this procedure. The identification of scyllo-inositol hexakisphosphate in soils and other environmental samples will allow its quantification in a range of environments, and facilitate research into the origins and function of this enigmatic compound.

Detailed information on soil organic P is fundamental to understanding biogeochemical cycles in both natural and managed ecosystems (Condron et al., 2004). One of the most intriguing aspects of soil organic P is the presence of inositol phosphates that seldom occur elsewhere in nature. The most common stereoisomeric forms are myo-inositol phosphate, which originate mainly in plant seeds, and scyllo-, D-chiro-, and neo-inositol phosphates also occur in varying proportions (Cosgrove, 1980). In particular, a large proportion of the soil organic P can occur as scyllo-inositol phosphates, yet the origin and function of this enigmatic group of compounds remain unknown (Turner et al., 2002). Free scyllo-inositol has often been detected in animals and plants, but rarely in phosphorylated forms (Posternak, 1965). Indeed, the report of a scyllo-inositol phospholipid in barley (Hordeum vulgare L. cv Himalaya) seed was the first of a phosphorylated scyllo-inositol in any biological tissue (Narasimhan et al., 1997).

Investigation of scyllo-inositol phosphates in soils is limited by a lack of suitable analytical techniques for their determination, because conventional procedures involve lengthy extraction, clean up, and chromatographic separation steps. Soil organic P can be characterized by alkaline extraction and solution 31P NMR spectroscopy (Condron et al., 1997), a procedure that has been improved recently by the adoption of a single-step NaOH–EDTA extraction, more accurate signal identification, and greater understanding of compound degradation during extraction and analysis (Cade-Menun and Preston, 1996; Makarov et al., 2002; Turner et al., 2003a). However, its use is limited for analysis of inositol phosphates due to poor resolution in the orthophosphate monoester region of the spectrum, although myo-inositol hexakisphosphate, the most abundant component of soil organic P, can now be accurately quantified in complex spectra with the aid of simple deconvolution software (Turner et al., 2003c).

The aim of this study was to develop a procedure for the determination of scyllo-inositol phosphates in soil extracts using alkaline extraction and solution 31P NMR spectroscopy. To achieve this we measured 31P NMR chemical shifts of synthetic scyllo-inositol phosphate standards in alkaline solution, then used hypobromite oxidation to demonstrate the presence of scyllo-inositol hexakisphosphate in alkaline extracts of soils.

MATERIALS AND METHODS

Solution 31P NMR Spectroscopy of Standard scyllo-Inositol Phosphates

All scyllo-inositol phosphate standards were synthesized and purified by the late Dr. Dennis Cosgrove, formerly of CSIRO Plant Industry, Canberra, Australia. No formal record of the procedures used to prepare these compounds exists, but probable procedures based on Cosgrove’s published methodologies are reported below.

The following samples were analyzed: a scyllo-inositol hexakisphosphate prepared by phosphorylation of scyllo-inositol by heating with polyporphoric acid, and purified from lower esters by ion-exchange chromatography (Cosgrove, 1966); a scyllo-inositol hexakisphosphate extracted from soil organic matter and presumably isolated by alkaline extraction and ion-exchange chromatography (Cosgrove, 1965); a scyllo-inositol pentakisphosphate and two scyllo-inositol tetrakisphosphate esters prepared by hydrolysis of scyllo-inositol hexakisphosphate by a wheat-bran phytase (Lim and Tate, 1973); a scyllo-inositol trisphosphate and a scyllo-inositol bisphosphate prepared using phytase isolated from a soil Pseudomonas (Irving and Cosgrove, 1971). All compounds were prepared as Ba salts, except the soil-derived scyllo-inositol hexakisphosphate, which was a free acid.

The scyllo-inositol phosphate standards were prepared for solution 31P NMR spectroscopy by mixing 10 mg of the Ba salt with 5 mL of deionized water and 5 mL of Amberlite IR-120 (H+ ) cation exchange resin (Sigma-Aldrich Co., St. Louis, MO). The resin was prepared initially by washing sequentially, and then mixed with the Ba salt and deionized water. The mixture was stirred for 24 h, and then filtered through a Whatman filter paper.
in deionized water, 1 M HCl, then again in deionized water. After swirling the slurry for several minutes, the mixture was filtered through a 0.2-μm cellulose-acetate syringe filter (Nalgene, Rochester, NY) and the resin washed three times with 5-mL aliquots of deionized water. The filtrate and washings were combined, made alkaline with a few drops of 1 M NaOH, then evaporated to dryness at 45°C.

For solution 31P NMR spectroscopy, each dried residue was redissolved in 0.8 mL of 1 M NaOH, 0.1 mL of 10 mg L⁻¹ K,HPO₄, and 0.1 mL of D₂O, and transferred to a 5-mm NMR tube. The NaOH ensured a pH > 13 (for consistent chemical shifts and optimum spectral resolution), the K,HPO₄ provided a reference signal, and the D₂O provided an NMR signal lock. Solution 31P NMR spectra were obtained using a Bruker Avance DRX 500 MHz spectrometer operating at 202.456 MHz for 31P and 100.134 MHz for 'H. Temperature was regulated at 27°C, and broadband proton decoupling was used for all samples. Samples were analyzed using a 5-μs pulse (45°), a delay time of 20.0 s, and an acquisition time of 0.8 s. The relatively long delay time ensured sufficient spin-lattice relaxation times by helping P nuclei to relax more rapidly (Cade-Menun et al., 2003). This technique oxidizes all organic matter except inositol phosphates. Briefly, 50 mL of extract was placed in a boiling tube, cooled in an ice bath, and 2 mL of pure Br (also cooled in an ice bath) added slowly in 0.5-mL aliquots. The mixture was left at room temperature for 1.5 h to allow oxidation to proceed, then boiled for 5 min to remove volatile reaction products. After cooling, 2 mL of concentrated NH₄OH was added to convert hypobromite to Br₂, and then the solution was acidified to pH < 3 with 10 M HCl to destroy carbonates. A small amount of concentrated NH₄OH was added to convert Br to Br₂, and the pH adjusted to >10 with 5 M NaOH. The solution was then frozen at −80°C.

The brominated and untreated extracts were lyophilized, and the residues ground to a fine powder. For solution 31P NMR spectroscopy, each freeze-dried extract (approximately 100 mg) was redissolved in 0.9 mL of 1 M NaOH and 0.1 mL of D₂O, and transferred to a 5-mm NMR tube. Brominated extracts were also analyzed by redissolving in 0.9 mL of a solution containing 1 M NaOH and 0.1 M EDTA (and 0.1 mL D₂O), which resulted in markedly improved resolution (see Results below). The pH of the redissolved samples varied slightly, but was always >13. Machine parameters were identical to those used for analysis of scyllo-inositol phosphate standards, except for a delay time of 1.0 s. This shorter delay time could be used because the extracts contained large concentrations of paramagnetic ions, which shorten spin-lattice relaxation times by helping P nuclei to relax more rapidly (Cade-Menun et al., 2002).

Chemical shifts were assigned to individual P compounds or functional groups based on literature reports (Turner et al., 2003a), with signal areas calculated by integration. Spectra were plotted using a 5-Hz line broadening, although additional spectra of brominated samples redissolved in NaOH plus EDTA were also plotted using a 1-Hz line broadening to conserve enhanced resolution.

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**Soil Extraction and Analysis**

Three lowland permanent pasture soils from the UK were extracted to investigate possible signals from scyllo-inositol phosphates. Soil physical and chemical properties are reported in Table 1. Recent studies have determined the P composition of these soils by solution 31P NMR spectroscopy (Turner et al., 2003b), and the concentrations of myo-inositol hexakisphosphate using a novel spectral deconvolution procedure (Turner et al., 2003c).

Phosphorus was extracted by shaking 5 g of soil with 100 mL of a solution containing 0.25 M NaOH and 0.05 M EDTA for 16 h at 20°C (Cade-Menun and Preston, 1996). The extracts were centrifuged at 10 000 × g for 30 min and half of each extract immediately frozen at −80°C. The remaining half was treated with hypobromite oxidation based on methodology described in Irving and Cosgrove (1981) and Suzumura and Kamatani (1993). This technique oxidizes all organic matter except inositol phosphates. Briefly, 50 mL of extract was placed in a boiling tube, cooled in an ice bath, and 2 mL of pure Br (also cooled in an ice bath) added slowly in 0.5-mL aliquots. The mixture was left at room temperature for 1.5 h to allow oxidation to proceed, then boiled for 5 min to remove volatile reaction products. After cooling, 2 mL of concentrated NH₄OH was added to convert hypobromite to Br₂, and then the solution was acidified to pH < 3 with 10 M HCl to destroy carbonates. A small amount of concentrated NH₄OH was added to convert Br to Br₂, and the pH adjusted to >10 with 5 M NaOH. The solution was then frozen at −80°C.

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Chemical shifts were assigned to individual P compounds or functional groups based on literature reports (Turner et al., 2003a), with signal areas calculated by integration. Spectra were plotted using a 5-Hz line broadening, although additional spectra of brominated samples redissolved in NaOH plus EDTA were also plotted using a 1-Hz line broadening to conserve enhanced resolution.

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**Table 1. Properties of the three soils used in the current study.**

<table>
<thead>
<tr>
<th>Soil</th>
<th>UK soil series</th>
<th>Location</th>
<th>USDA class</th>
<th>Top soil property</th>
<th>Soil composition</th>
<th>Soil pH (water)</th>
<th>Oxalate Fe, g kg⁻¹</th>
<th>myo-inositol hexakisphosphate, mg P kg⁻¹ soil†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Denbigh</td>
<td>Camelford, Cornwall</td>
<td>Dystrochrepts</td>
<td>Clay</td>
<td>1.54</td>
<td>45.0</td>
<td>4.5</td>
<td>189</td>
</tr>
<tr>
<td>2</td>
<td>Brickfield</td>
<td>Llangefni, Anglesey</td>
<td>Haquepepts</td>
<td>Clay</td>
<td>1.11</td>
<td>4.8</td>
<td>5.3</td>
<td>144</td>
</tr>
<tr>
<td>3</td>
<td>Fladbury</td>
<td>Glastonbury, Somerset</td>
<td>Fluvaquents</td>
<td>Clay</td>
<td>1.98</td>
<td>5.0</td>
<td>12.9</td>
<td>163</td>
</tr>
<tr>
<td>L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Determined in NaOH–EDTA extracts by solution 31P NMR spectroscopy and spectral deconvolution (Turner et al., 2003c).

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**Fig. 1. Structure of scyllo-inositol hexakisphosphate in solution:** (a) the all equatorial structure at pH < 9.0, (b) the all axial structure at pH > 11.0 (Volkmann et al., 2002).
RESULTS

Conformation of scyllo-Inositol Hexakisphosphate at pH > 13

Of the nine possible inositol stereoisomers, scyllo-inositol is the most simple, because all hydroxyl groups are structurally equivalent, being either all axial or all equatorial depending on solution pH (Fig. 1). Thus, scyllo-inositol differs from the myo isomer by the orientation of only a single hydroxyl group. It was reported that scyllo-inositol hexakisphosphate displays an equatorial structure at pH < 9 and an all-axial structure at pH > 11; between these pH values there is probably dynamic equilibrium between the two conformations (Volkmann et al., 2002). The pH of the samples analyzed by solution $^{31}$P NMR spectroscopy in our experiments was always >13, so scyllo-inositol hexakisphosphate was in the all-axial conformation. Based on evidence of the conformation of lower myo-inositol phosphate esters at different pH values (Barrientos and Murthy, 1996), it seems likely that the lower scyllo-inositol phosphate esters display similar conformational changes to scyllo-inositol hexakisphosphate at different pH values, although there is no direct evidence for this.

Solution $^{31}$P NMR Spectroscopy of scyllo-Inositol Phosphates

Solution $^{31}$P NMR spectra of scyllo-inositol phosphate standards are shown in Fig. 2. Details of contributing P groups and chemical shifts standardized to that of orthophosphate at 6.0 ppm are also reported (Table 2). Signals for scyllo-inositol phosphate regioisomers not analyzed here were calculated from data for synthetic compounds reported in Chung et al. (1999) (Table 2).

The synthetic scyllo-inositol hexakisphosphate gave a single signal at 4.163 ppm (Fig. 2), and a similar signal at 4.155 ppm was detected for the same compound extracted from soil organic matter (not shown). This single signal arises because the chemical environments around all six phosphate moieties in scyllo-inositol hexakisphosphate are identical. In both spectra, small signals at approximately 3.99, 4.24, and 4.70 ppm indicated the presence of trace amounts of scyllo-inositol pentakisphosphate (see below).

There is only one form of scyllo-inositol pentakisphosphate, here termed scyllo-inositol (1,2,3,4,5) pentakisphosphate for simplicity (labeling is arbitrary as all phosphates are structurally identical). Signals from this compound were detected at 3.994, 4.251, and 4.709 ppm in the ratio 2:1:2. A small signal at 4.179 ppm indicated a trace of scyllo-inositol hexakisphosphate, while other small signals between 4.8 and 5.6 ppm indicated the presence of lower order scyllo-inositol phosphates.

The two scyllo-inositol tetrakisphosphate esters gave markedly different spectra. One gave a single signal at 5.102 ppm and was thus identified as scyllo-inositol (1,2,3,5) tetrakisphosphate (Chung et al., 1999). The other gave signals at 4.276, 4.872, and 5.299 ppm in the ratio 1:2:1, and was thus identified as scyllo-inositol (1,2,3,5) tetrakisphosphate (Chung et al., 1999).
Table 2. Solution $^{31}$P NMR chemical shifts of all possible forms of scyllo-inositol phosphates in alkaline solution. Values were determined by spectroscopy of compounds purified by the late Dr. Dennis Cosgrove, or calculated from literature values (Chung et al., 1999).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical shift t (ppm)</th>
<th>Number of P groups</th>
<th>Contributing P groups</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>scyllo-1(1,2,3,4,5,6)P$_6$</td>
<td>4.137</td>
<td>6</td>
<td>P$_1$, P$_2$, P$_3$, P$_4$, P$_5$, P$_6$</td>
<td>This study</td>
</tr>
<tr>
<td>scyllo-1(1,2,3,4,5)P$_5$</td>
<td>3.979</td>
<td>2</td>
<td>P$_2$, P$_4$</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>4.235</td>
<td>1</td>
<td>P$_3$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.693</td>
<td>2</td>
<td>P$_1$, P$_2$, P$_4$, P$_5$</td>
<td>This study</td>
</tr>
<tr>
<td>scyllo-1(1,2,4,5)P$_4$</td>
<td>5.065</td>
<td>4</td>
<td>P$_2$, P$_3$</td>
<td>This study</td>
</tr>
<tr>
<td>scyllo-1(1,2,3,5)P$_4$</td>
<td>4.248</td>
<td>1</td>
<td>P$_1$, P$_3$</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>4.844</td>
<td>2</td>
<td>P$_1$, P$_3$</td>
<td></td>
</tr>
<tr>
<td>scyllo-1(1,2,3,4)P$_4$</td>
<td>4.127</td>
<td>2</td>
<td>P$_2$, P$_3$</td>
<td>Chung et al. (1999)</td>
</tr>
<tr>
<td>scyllo-1(1,2,3)P$_3$</td>
<td>4.987</td>
<td>2</td>
<td>P$_2$, P$_4$</td>
<td></td>
</tr>
<tr>
<td>scyllo-1(1,2,4)P$_3$</td>
<td>4.723</td>
<td>1</td>
<td>P$_5$</td>
<td></td>
</tr>
<tr>
<td>scyllo-1(1,2,4)P$_2$</td>
<td>5.550</td>
<td>2</td>
<td>P$_1$, P$_3$</td>
<td>Chung et al. (1999)</td>
</tr>
<tr>
<td>scyllo-1(1,3,5)P$_3$</td>
<td>5.471</td>
<td>1</td>
<td>P$_4$</td>
<td></td>
</tr>
<tr>
<td>scyllo-1(1,3)P$_2$</td>
<td>5.477</td>
<td>2</td>
<td>P$_1$, P$_2$, P$_3$</td>
<td>Chung et al. (1999)</td>
</tr>
<tr>
<td>scyllo-1(1,4)P$_2$</td>
<td>5.211</td>
<td>3</td>
<td>P$_1$, P$_2$, P$_5$</td>
<td>Chung et al. (1999)</td>
</tr>
<tr>
<td>scyllo-1(1,4)P$_1$</td>
<td>5.468</td>
<td>2</td>
<td>P$_1$, P$_2$</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>5.277</td>
<td>2</td>
<td>P$_1$, P$_3$</td>
<td>Chung et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>5.157</td>
<td>2</td>
<td>P$_1$, P$_4$</td>
<td>Chung et al. (1999)</td>
</tr>
</tbody>
</table>

† Corrected for orthophosphate chemical shift at 6.0 ppm.
‡ Chemical shifts calculated from Chung et al. (1999) are standardized from values determined at pH 10 relative to that of scyllo-inositol hexakisphosphate at 4.137 ppm.

The scyllo-inositol trisphosphate gave signals at 4.760 and 5.587 ppm in the ratio 1:2, and was thus identified as scyllo-inositol (1,2,3) trisphosphate (Chung et al., 1999). The scyllo-inositol bisphosphate gave a single signal at 5.506 ppm, which based on the signals for the three possible bisphosphate esters was identified as the scyllo-inositol (1,2) bisphosphate, rather than the (1,3) or (1,4) esters (Chung et al., 1999).

Identification of scyllo-Inositol Phosphates in Alkaline Soil Extracts

Solution $^{31}$P NMR spectra of soil NaOH–EDTA extracts are shown in Fig. 3, with the proportions of individual compounds reported in Table 3. Brominated extracts redissolved in NaOH gave poorly resolved spectra, with significant line broadening that obscured all but the strongest signals (Fig. 3). However, when extracts were redissolved in NaOH plus EDTA, spectral resolution improved markedly, as demonstrated by the expanded spectra plotted with 1 Hz line broadening. Signals from DNA between –0.12 and –0.23 ppm remained unchanged in all three untreated extracts following bromination, indicating that DNA was at least partly degraded by the bromination procedure. This is in contrast to a previous literature report (Nanny and Minear, 1997). However, pyrophosphate signals at –4.3 ppm were relatively unaltered by bromination, suggesting that simple P speciation by molybdate colorimetry cannot be used to determine the inositol phosphate fraction in brominated extracts.

Signals upfield of orthophosphate at approximately 6.6 and 6.8 ppm in all three untreated extracts have been previously assigned to compounds similar in structure to aromatic orthophosphate diesters (Bedrock et al., 1994; Turner et al., 2003a). However, these signals remained relatively unchanged by bromination, indicating that they represent unidentified inositol phosphates. More detailed studies are required to unambiguously identify these signals, although a sample of mixed neo-inositol pentakisphosphates gave similar signals in this region of the spectrum (data not shown).

Signals from DNA between –0.12 and –0.23 ppm in untreated extracts were hardly detectable in brominated extracts, indicating that DNA was at least partly degraded by the bromination procedure. This is in contrast to a previous literature report (Nanny and Minear, 1997). However, pyrophosphate signals at –4.3 ppm were relatively unaltered by bromination, suggesting that simple P speciation by molybdate colorimetry cannot be used to determine the inositol phosphate fraction in brominated extracts.

DISCUSSION

The identification of the strong signal at approximately 4.2 ppm in alkaline soil extracts as scyllo-inositol hexakisphosphate is significant, because this signal is prominent in solution $^{31}$P NMR spectra of extracts of...
many different types of soils reported in the literature. It is impossible to rule out the contribution of choline phosphate to this signal without bromination, although the rapid turnover of labile orthophosphate monoesters in soil (Bowman and Cole, 1978) suggests that choline phosphate is unlikely to be quantitatively important in most cases. It is also unlikely to originate from the breakdown of phosphatidyl choline during extraction and analysis, because this leads to β-glycerophosphate (4.81 ppm) and phosphatidic acid (5.15 ppm) rather than choline phosphate (Turner et al., 2003a). Further, signals from choline phosphate and scyllo-inositol hexakisphosphate are not exactly coincident, occurring at 4.02 and 4.14 ppm, respectively, when corrected for the

**Table 3.** Proportions of P compounds determined by alkaline extraction and solution 31P NMR spectroscopy of three temperate pasture soils. Extracts were analyzed without pretreatment or following hypobromite oxidation to destroy all organic matter except inositol phosphates.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Chemical shift ppm</th>
<th>Untreated</th>
<th>Brominated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil 1</td>
<td>Myo-inositol hexakisphosphate</td>
<td>4.5, 4.7, 5.0, 5.9</td>
<td>18.9</td>
</tr>
<tr>
<td></td>
<td>Scyllo-inositol hexakisphosphate</td>
<td>4.2</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>Orthophosphate</td>
<td>6.2</td>
<td>22.7</td>
</tr>
<tr>
<td></td>
<td>Pyrophosphate</td>
<td>-4.3</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>DNA</td>
<td>-0.12 to -0.24</td>
<td>3.0</td>
</tr>
<tr>
<td>Soil 2</td>
<td>Myo-inositol hexakisphosphate</td>
<td>4.5, 4.7, 5.0, 5.9</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>Scyllo-inositol hexakisphosphate</td>
<td>4.2</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Orthophosphate</td>
<td>6.2</td>
<td>36.4</td>
</tr>
<tr>
<td></td>
<td>Pyrophosphate</td>
<td>-4.3</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>DNA</td>
<td>-0.12 to -0.24</td>
<td>3.0</td>
</tr>
<tr>
<td>Soil 3</td>
<td>Myo-inositol hexakisphosphate</td>
<td>4.5, 4.7, 5.0, 5.9</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>Scyllo-inositol hexakisphosphate</td>
<td>4.2</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>Orthophosphate</td>
<td>6.2</td>
<td>38.9</td>
</tr>
<tr>
<td></td>
<td>Pyrophosphate</td>
<td>-4.3</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>DNA</td>
<td>-0.12 to -0.24</td>
<td>4.9</td>
</tr>
</tbody>
</table>

ND, not detected.

† Based on approximate chemical shift values (Fig. 3).

‡ Values calculated by multiplying by six the signal from the phosphate at the C2 position on the inositol ring at approximately 5.9 ppm (Turner, 2004).
chemical shift of orthophosphate at 6.0 ppm. It therefore seems certain that scylo-inositol hexakisphosphate represents a significant component of soil organic P (6-10% NaOH-EDTA extractable P in the three soils analyzed here), confirming previous studies that have identified scylo-inositol phosphates in soils using conventional chromatography (Cosgrove, 1980; reviewed recently by Turner et al., 2002).

Identification and quantification of scylo-inositol hexakisphosphate in soil extracts using solution 31P NMR spectroscopy is simplified by the strength of the signal, which arises because the environments around all six phosphates are chemically identical. This is in contrast to the four signals from myo-inositol hexakisphosphate in the ratio 1:2:2:1, although it is now possible to quantify these relatively easily using spectral deconvolution software (Turner et al., 2003c). Chemical shifts of lower order scylo-inositol phosphate esters are reported, but were not identified in the soils analyzed here. The presence of scylo-inositol pentakisphosphate would be most readily detected by the signal at 3.99 ppm from the C2 and C4 phosphates (Fig. 2), because this would be well separated from the main envelope of signals in the orthophosphate monoester region. However, based on literature information it seems unlikely that the pentakisphosphates would occur in sufficient quantities to permit detection by solution 31P NMR spectroscopy.

In poorly resolved spectra, pretreatment of samples by hypobromite oxidation will markedly improve the accuracy of scylo-inositol hexakisphosphate quantification, and eliminate the possible contribution of choline phosphate. The technique also markedly improves spectral resolution, providing that extracts are redissolved in NaOH plus EDTA, rather than NaOH alone. The poor spectral resolution of brominated extracts redissolved in NaOH alone is probably due to destruction of EDTA during hypobromite oxidation, which allows P nuclei to come into close proximity with paramagnetic ions. Reintroducing EDTA prevents this interaction by chelating paramagnetic ions, yet maintains them in solution to allow short delay times to be used. The inclusion of EDTA in the NMR tube is therefore likely to be important when analyzing other types of samples that contain interfering paramagnetic ions, such as water or organic anion extracts of soils.

The ability to quantify scylo-inositol hexakisphosphate in soil extracts will facilitate more detailed and widespread investigation of this intriguing organic phosphate. The origins of scylo-inositol hexakisphosphate in soils remain unclear, but the fact that it has been detected only in soils and aerobically digested sewage sludge suggests a probable microbial source (Cosgrove, 1980). Indeed, an isomer of myo-inositol hexakisphosphate, subsequently shown by Cosgrove to be scylo-inositol hexakisphosphate, was detected after a sand-clay mixture containing inorganic and organic nutrients was incubated with soil microorganisms (Caldwell and Black, 1958). However, it remains unclear whether microbes directly synthesize scylo-inositol hexakisphosphate from carbohydrate precursors, or epimerize it from myo-inositol hexakisphosphate. Whatever the mechanism involved, the ecological function of scylo-inositol hexakisphosphate in soils is completely unknown. Future research should focus on quantifying the concentrations of scylo-inositol hexakisphosphate in soils from a wide range of environments, and investigating its biochemical origin and function in soil.

ACKNOWLEDGMENTS

The authors thank Dr. Alex Blumenfeld for analytical support, and Dr. Barbara J. Cade-Menun, and Dr. Michael F. L’Annunziata for comments on the manuscript.

REFERENCES

Narasimhan, B., G. Pliska-Matyshak, R. Kinnard, S. Cartensen, M.A.
Suzumura, M., and A. Kamatani. 1993. Isolation and determination
of inositol hexaphosphate in sediments from Tokyo Bay. Geochim.
Turner, B.L. 2004. Optimizing phosphorus characterization in animal
manures by solution phosphorus-31 nuclear magnetic resonance
Turner, B.L., M. Papház, P.M. Haygarth, and I.D. McKelvie. 2002.
Inositol phosphates in the environment. Philos. Trans. R. Soc.
nuclear magnetic resonance spectral assignments of phosphorus

Turner, B.L., N. Mahieu, and L.M. Condron. 2003b. The phosphorus
composition of temperate pasture soils determined by NaOH–
EDTA extraction and solution 31P NMR spectroscopy. Org. Geo-
chem. 34:1199–1210.
Turner, B.L., N. Mahieu, and L.M. Condron. 2003c. Quantification
of myo-inositol hexakisphosphate in alkaline soil extracts by solution
31P NMR spectroscopy and spectral deconvolution. Soil Sci.
Volkmann, C.J., G.M. Chateauneuf, J. Pradhan, A.T. Bauman, R.E.
Brown, and P.P.N. Murthy. 2002. Conformational flexibility of
inositol phosphates: Influence of structural characteristics. Tetrahe-
dron Lett. 43:4853–4856.
Wrenshall, C.L., and W.J. Dyer. 1941. Organic phosphorus in soils:
II. The nature of the organic phosphorus compounds. A. Nucleic