Analysis of Inorganic Polyphosphates by Capillary Gel Electrophoresis

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ABSTRACT

This paper describes the development of a method that uses Capillary Gel Electrophoresis (CGE) to analyze mixtures of inorganic polyphosphate ((P$_i$)$_n$). Resolution of (P$_i$)$_n$ on the basis of $n$, the number of residues of dehydrated phosphate, is accomplished by CGE using capillaries filled with solutions of poly($N,N$-dimethylacrylamide) (PDMA) and indirect detection by the UV-absorbance of a chromophore, terephthalate, added to the running buffer. The method is capable of resolving peaks representing (P$_i$)$_n$ with $n$ up to ~70; preparation and use of authentic standards enables the identification of peaks for (P$_i$)$_n$ with $n = 1 - 10$. The main advantages of this method over previously reported methods for analyzing mixtures of (P$_i$)$_n$ (slab gel electrophoresis, free solution capillary electrophoresis, CGE using polyacrylamide) are its resolution, convenience, and reproducibility; gel-filled capillaries are easily re-generated by pumping in fresh, low-viscosity solutions of PDMA. The resolution is comparable to that of ion-exchange chromatography and detection of (P$_i$)$_n$ by suppressed conductivity. The method is useful for analyzing (P$_i$)$_n$ generated by the dehydration of P$_i$ at low temperature (125 - 140 °C) with urea, in a reaction that may have been important in prebiotic chemistry. The method should also be useful for characterizing mixtures of other anionic, oligomeric or polymeric species without an intrinsic chromophore (e.g., sulfated polysaccharides, oligomeric phospho-diesters).
INTRODUCTION

This paper describes a technique for the analysis of mixtures of inorganic oligo- and poly-phosphates, \((P_i)_n\), by capillary gel electrophoresis. Samples of condensed inorganic phosphate consist of mixtures of oligomeric and polymeric species of composition \((P_i)_n\), where \(n\) is the number of residues of inorganic phosphate. Mixtures of \((P_i)_n\) can have a wide range in \(n\); \((P_i)_n\) with estimated values of \(n\) as high as 1000 have been reported.\(^1\)

The analytical method developed here characterizes samples of \((P_i)_n\) at high resolution by separating and detecting each species in a mixture.

Species of \((P_i)_n\) in aqueous solution are anionic, and differ from each other in the number of residues of condensed phosphate and in their net negative electrostatic charge. Capillary electrophoresis (CE), in its most straightforward mode of operation – capillary zone electrophoresis (CZE): that is, electrophoresis of analytes through free solution, combined with optical detection of chromophoric analytes – cannot resolve and detect \((P_i)_n\). We developed a method that addresses the two problems of i) separating each species of \((P_i)_n\) in a mixture and ii) detecting and quantifying each \((P_i)_n\). Capillary gel electrophoresis (CGE) using capillaries filled with aqueous solutions of poly(\(N,N\)-dimethylacrylamide) (PDMA) resolved oligomers of structure \((P_i)_n\) in order of their size. Addition of the chromophoric anion, terephthalate (\(C_6H_4-1,4-(CO_2^-)\)_2, abbreviated as \(TP^2^-\)), to the running buffer enabled the detection of separated \((P_i)_n\) by indirect UV absorbance.

We demonstrated the ability of the method to characterize samples of \((P_i)_n\) by resolving several different mixtures of \((P_i)_n\). We also compared the mobilities of components in mixtures with those of authentic standards. Analysis of the areas of peaks
in electropherograms obtained by indirect detection allowed us to quantify the relative concentration of each species in a mixture of \((P_i)_n\). In addition to analyzing commercially available samples of \((P_i)_n\), prepared by the thermal dehydration of \(P_i (\geq 220 ^\circ C)\), we showed that this method can analyze \((P_i)_n\) generated by dehydration reactions that might have occurred on the prebiotic earth, and thus might possibly have been involved in the chemical origins of life.\(^2\-^4\)

**Motivation.**

Investigation of the chemistry of \((P_i)_n\) depends on convenient methods for characterizing mixtures of \((P_i)_n\) that vary in chain length, and on methods for characterizing the structures of \((P_i)_n\) in solution. Our primary motivation to develop a new method for analyzing mixtures of \((P_i)_n\) was to explore syntheses and reactivities of \((P_i)_n\) relevant to the chemical origins of life.\(^2\-^3,^5\) This method should also be useful for investigating the biochemistry of \((P_i)_n\),\(^6\-^8\) and for developing \((P_i)_n\) as a reagent in synthetic chemistry. In addition, the method may be useful in applications for quality control: \((P_i)_n\) is a component of many commercial materials (e.g., fertilizers, food products, detergent formulations, building materials).\(^9\)

**\((P_i)_n\) as a Molecular Fossil; \((P_i)_n\) and ATP.** Adenosine triphosphate (ATP) and \((P_i)_n\) have an essential functional group in common – residues of dehydrated phosphate connected by phosphoanhydride bonds. Reactions of ATP (and other nucleoside triphosphates) involving the triphosphate moiety – the transfer of phosphate, pyrophosphate, or nucleotide diphosphate groups – are ubiquitous in biochemistry.\(^10\-^12\) These reactions function in the activation of \(-\text{OH}\) groups (i.e., the biological equivalent
of tosyl chloride) essential in metabolic pathways and the biosynthesis of nucleic acids, poly-saccharides, and proteins from monomeric species. By serving as a donor of phosphate groups, ATP is also involved in the modification of the properties of a wide range of biochemical substrates (e.g., to the side chains of Tyr, Ser and Thr residues in proteins, and to carbohydrates). The electrostatic charge and hydrogen-bonding interactions of phosphate groups contribute to the solubility and membrane-permeability of phosphorylated species; phosphate groups also provide a handle for molecular recognition. That (P$_i$)$_n$ is simpler in composition than ATP, but in principle can provide the same chemical function, has led to the suggestion that (P$_i$)$_n$ is a *molecular fossil* – a species important in the origin of life – and a precursor to ATP. The implicit hypothesis of this idea is that (P$_i$)$_n$ served a role in prebiotic chemistry related to that of ATP in biochemistry.

**(P$_i$)$_n$ in Chemical Origins of Life (COoL).** The plausible generation and reactivity of (P$_i$)$_n$ in COoL (i.e., the prebiotic chemistry leading to self-replicating systems in a “pre-RNA” or “RNA world”) has been raised before. Previous studies demonstrated the synthesis of oligophosphates at conditions assumed plausible for prebiotic earth. Heating orthophosphate (Na$^+$ or Ca$^{2+}$ salts) to 160 °C led to the conversion to P$_2$ and P$_3$ (with yields up to 50% and 30% respectively). Heating (NH$_4$)$_2$HPO$_4$ to 100 °C, in an excess of urea under a N$_2$ atmosphere, generated (P$_i$)$_n$ with $n > 5$ at yields > 95%. Orgel and others tested the hypothesis that (P$_i$)$_n$ could enable the polymerization of amino acids and nucleosides. Solid-state reactions of glycine in the presence of (P$_i$)$_n$ and imidazole led to distributions of oligoglycine (including peptides higher than (Gly)$_{10}$) and diketopiperazine. Reaction of the phosphorimidazolide of
adenosine (generated by reaction of imidazole and ATP), catalyzed by montmorillonite, led to oligonucleotide products containing more than 50 monomers. We were interested in exploring plausible prebiotic conditions for the dehydration of inorganic P in greater detail, and needed a method of analysis for \((P_i)_n\) that was superior to those so far reported.

Exploration of the reactivity of \((P_i)_n\), in either prebiotic conditions or in applications in synthetic chemistry, remains incomplete. Demonstrations of the reactivity of \((P_i)_n\) towards organic compounds are surprisingly limited; synthesis of phosphate esters typically use P(III) or P(V) reagents activated with P-Cl or P-N bonds. Investigation of the reactivity of trimetaphosphate (\(cyclo-P_3\)) in aqueous solution discovered the ability of ammonia to catalyze phosphorylations of \(\alpha\)-hydroxyl groups of aldose sugars by \(cyclo-P_3\). Ammonolysis of \(cyclo-P_3\) generated amido-triphosphate (AmTP, \(\text{NH}_2(\text{PO}_3^-)_2(\text{PO}_3^{2-})\)). Analysis of reactions of AmTP with glycolaldehyde (as well as glyceraldehyde, aldotetroses, and aldofuranoses) in aqueous solution demonstrated that AmTP is a better phosphorylating agent than \(cyclo-P_3\). The initial step of the proposed mechanism is the condensation of AmTP with the aldehyde group, and generation of an intramolecular phosphorylating agent. The ultimate products were \(\alpha\)-hydroxy phosphate monoesters (e.g., 2-glyceraldehyde phosphate) or cyclic diesters (e.g., 1,2-cyclophosphate of threose). Products did not however include oligomeric or polymeric species. Investigation of non-enzymatic (but perhaps biomimetic) reactions that transfer phosphate residues from linear or cyclic \((P_i)_n\) to organic compounds will be necessary for further development of hypotheses about the involvement of \((P_i)_n\) in the origin of life, as well as hypotheses concerning the first oligomers and polymers of prebiotic significance.
**\((P_i)_n\) in Biochemistry/Metabolism.** The presence of \((P_i)_n\) in cells of all organisms establishes the importance of the biochemistry of \((P_i)_n\).\textsuperscript{6-8,36-37} The investigation of \((P_i)_n\) and its function in phosphate metabolism are largely based on the study of enzymes that catalyze reactions that synthesize or utilize \((P_i)_n\).\textsuperscript{38-42} Polyphosphate kinases (PPK) catalyze the reversible transfer of phosphate residues from \((P_i)_n\) to ADP (Eq. 1), and in some cases from \((P_i)_n\) to GDP.\textsuperscript{39}

\[
(P_i)_n + ADP \rightleftharpoons (P_i)_{n-1} + ATP
\]  

(1)

The analysis of \((P_i)_n\) by Polyacrylamide Gel Electrophoresis (PAGE, discussed below) in reactions synthesizing ATP (catalyzed by PPK from *P. shermanii*) showed decreasing amounts of \((P_i)_n\) in the reaction mixture over time, but did not show changes in the distribution of chain lengths. Remaining \((P_i)_n\) in the mixture had \(n > 750\), and ladders of \((P_i)_n\) of decreasing chain length (i.e., bands for \((P_i)_{n-1}\), \((P_i)_{n-2}\), etc.) did not develop.\textsuperscript{43} The results led to the conclusion that PPK catalyzes the transfer of phosphate from \((P_i)_n\) to ADP in a *processive* mechanism (i.e., the rate-limiting step is association of \((P_i)_n\) to the enzyme; a chain of \((P_i)_n\) phosphorylates multiple equivalents of ADP prior to dissociation from PPK).\textsuperscript{39,43} Further background material on enzymes catalyzing reactions of \((P_i)_n\)\textsuperscript{44-45} and on other potential roles of \((P_i)_n\) in biology is available in the Supporting Information.

**Previously Reported Methods of Analysis of Mixtures of \((P_i)_n\).**

**Bulk Properties of Solutions of \((P_i)_n\).** Properties such as density, viscosity, conductivity, index of refraction, and vapor pressure of aqueous solutions, can be used to
calibrate and estimate the extent of polymerization of a sample of \((P_i)_n\). These methods provide only a crude level of characterization, and are unable to identify or quantify the relative amounts of different \((P_i)_n\) in mixtures.\(^{46}\)

**Estimating Averages Values of \(n\) by Analyzing Terminal Groups.** Analysis of aqueous solutions of \((P_i)_n\) by pH titration with NaOH is useful for estimating the average chain length \((\bar{n})\) in samples of linear \((P_i)_n\). Titration of the second acidic hydrogen of terminal phosphate groups (\(pK_a \sim 7\)) enables the measurement of the number of terminal groups, \(N_t\), in a mixture.\(^{47}\) Values of \(\bar{n}\) are estimated by the quantity \(2(N)(N_t)^{-1}\), where \(N\) is the number of total phosphate residues in the sample; the value of \(N\) can be determined by colorimetrically.\(^{48-49}\) Estimates for \(N_t\) can also be obtained by assay with phosphoglucomutase\(^{50-51}\) or by \(^{31}\)P NMR.\(^{52}\) Chemical shifts of terminal phosphate groups (\(\delta \sim 5.4 – 6.8\) ppm, chemical shifts referenced to 85% phosphoric acid) are well resolved from those of internal phosphate groups (\(\delta \sim 21\) ppm).

**Resolution by Polyacrylamide Gel Electrophoresis (PAGE).** The resolution of PAGE enables the characterization of distributions of \((P_i)_n\), for \(n \sim 2 - 450\). The technique involves the separation of \((P_i)_n\) by electrophoresis through gels of cross-linked polyacrylamide (typical runs require \(\geq 3\) hours), and the detection of \((P_i)_n\) by staining the gels with a cationic dye, Toluidine Blue O (TBO)\(^{1,38,43}\); autoradiography can detect species of \((P_i)_n\) synthesized from \(^{32}\)P-ATP.\(^{45}\) Gels consisting of 20% polyacrylamide resolve \((P_i)_n\) into bands, for up to \(n \sim 30\). Gels with lower amounts of polyacrylamide (2 - 10%) allow for the approximation of chain lengths of \((P_i)_n\) for \(n > 100\) residues (up to 450).\(^1\) The relationship between the log of the chain length of a species and the distance

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\(^{48-49}\) Estimates for \(N_t\) can also be obtained by assay with phosphoglucomutase or by \(^{31}\)P NMR.

\(^{50-51}\) Chemical shifts of terminal phosphate groups (\(\delta \sim 5.4 – 6.8\) ppm, chemical shifts referenced to 85% phosphoric acid) are well resolved from those of internal phosphate groups.

\(^{52}\) Gels consisting of 20% polyacrylamide resolve \((P_i)_n\) into bands, for up to \(n \sim 30\). Gels with lower amounts of polyacrylamide (2 - 10%) allow for the approximation of chain lengths of \((P_i)_n\) for \(n > 100\) residues.
migrated (in units of $R_f$, relative to bromphenol blue) is linear. Preparative scale PAGE can be used to obtain partially-purified standards.\(^53\)

The disadvantages to PAGE are the difficulty of the experiments and the time required for analysis. We found it difficult to cast 20% gels that are homogenous and provide reproducible resolution; to run a gel required several hours (separation, staining and destaining). Although staining with TBO enables the detection of $(P_i)_n$ in amounts of $2 – 20 \mu g$,\(^1\) the quantitative determination of $(P_i)_n$ resolved in gels has not been demonstrated. TBO is not a sensitive stain for $P_1 - P_3$.\(^51\)

**Anion-exchange Chromatography.** The separation of $(P_i)_n$ by anion exchange chromatography is useful both for analyzing mixtures of $(P_i)_n$ and for preparing samples of purified $(P_i)_n$. The best results for the chromatographic resolution of $(P_i)_n$ are chromatograms showing up to $\sim 50$ resolved peaks, in runs requiring less than 30 minutes.\(^54-55\) The analytical method requires HPLC instrumentation with a suppressed conductivity detector and an on-line KOH gradient generator (for minimizing the amount of $CO_2/CO_3^{2-}$ adsorbed from atmosphere). Distinguishing samples containing $(P_i)_n$ with $n > 45$ is difficult by this method, and the resolution of species with $n \sim 100$ has not been demonstrated. Gradients of $Cl^-$ or $NO_3^-$ in buffered solutions can also separate $(P_i)_n$ over anion exchange chromatography columns.\(^56-58\) Alternative methods of detection include post-column derivatization with reagents for colorimetric detection ($Fe(ClO_4)_3$ or $H_2SO_4$ at $90 ^\circ C$, ammonium molybdate, hydrazine sulfate).\(^59\) These types of experiments, while useful for preparatory scale separation of $(P_i)_n$ up to $n \sim 30$, require too much time ($\geq 6$ hours) to be useful for the analysis of large numbers of samples.
Other Methods. Other qualitative or semi-quantitative methods used to analyze mixtures of \((P_i)_n\) include paper chromatography\(^{23,46}\), \(^{31}\)P NMR\(^{52,60-64}\) and ESI-MS\(^{65}\).

Capillary Electrophoresis and the Analysis of Ions.

Capillary electrophoresis separates and resolves analytes based on the differences in electrophoretic mobility, \(\mu\). The characteristic \(\mu\) of an analyte is determined by its electrostatic charge (leading to migration in an applied field) and its hydrodynamic radius (leading to viscous drag opposing migration). The electrophoretic mobility is often expressed by Eq. 2. In this equation, \(C_p\) is a constant, \(z\) is the electrostatic charge, \(m\) is the mass of the ion, and \(\alpha\) depends on the conformation of the molecule (and other factors that relate \(m\) to an apparent hydrodynamic radius).

\[
\mu = C_p \frac{z}{m^\alpha}
\]  

(2)

Values of \(\alpha\) in models for hard spheres and free-draining coils are 1/3 and 1 respectively; for most globular proteins, \(\alpha\) is 2/3.

Mobilities of \((P_i)_n\) and DNA. While the conformational structure of \((P_i)_n\) in solution has not been determined (i.e., \(\alpha\) for \((P_i)_n\) in Eq. 2 is unknown), we expected the relationship between the mobility and length of \((P_i)_n\) to be similar to that of single-stranded DNA. The relationship between the length of a single-stranded DNA molecule of \(N\) nucleotides and its mobility in free solution is expressed in Eq. 3; \(z_m\) and \(m_m\) are the charge and mass of mononucleotides comprising oligonucleotide chains.
The mobilities of DNA with \( N \) and \( N + 1 \) nucleotides are exactly equal when \( \alpha = 1 \), and are approximately the same when \( \alpha = 2/3 \) for large \( N \). Electrophoresis through free solution therefore cannot separate different lengths of single-stranded DNA solely on the basis of their size.\(^{66} \) Samples of \((P_i)_n\) are usually mixtures of ions that differ in the number of residues of phosphate, \( n \). Since both the amount of negative charge and hydrodynamic drag increase with \( n \), we expected poor resolution in the analysis of \((P_i)_n\) by CZE.\(^{67} \)

**Mobilities in Gels.** In CGE, analytes migrate through a porous gel, composed of either a cross-linked polymer or a solution of an entangled polymer network. Gel electrophoresis separates analytes unresolvable by CZE (e.g., DNA, complexes of sodium dodecyl sulfate with denatured protein) on the basis of migration through pores of the gel, in which the radius of the pores is comparable to the radius of migrating species. Expressions for the mobility of analytes through a polymer network, based on the Ogston model\(^{66,68} \) or reptation model\(^ {69} \), are given in Eq. 4 and Eq. 5 respectively. In Eq. 4,

\[
\mu_{\text{gel}} = \mu_0 \exp(-KC(r + R_g)^2)
\]

and

\[
\mu = C_p \frac{z}{m^\alpha} \sim \frac{N}{N^2} \sim \frac{1}{N}
\]
µ₀ is the mobility in free solution, K is a constant, C is the concentration of the polymer comprising the gel, r is the thickness of a polymer strand, and \( R_g \) is the radius of gyration of the analyte. In Eq. 5, \( N \) is proportional to the size of the size of molecule (e.g., number of nucleotides in DNA). Both models predict mobilities that depend on the chain length. The mobilities of \((P_i)_n\) through a porous gel decrease with increasing hydrodynamic radius. We therefore expected CGE to resolve mixtures of \((P_i)_n\) in order of number of phosphate residues, \( n \).

**Prior Use of CGE to Analyze \((P_i)_n\)**. Previously reported methods using capillaries coated and filled with linear polyacrylamide, prepared by filling capillaries with solutions of acrylamide and polymerizing *in situ*, resolved \((P_i)_n\) in order of \( n \).\(^{70-71}\) We, and others, found that the gel-filled capillaries prepared this way had short lifetimes\(^{72}\), due to either the formation of air bubbles or degradation of the gel.\(^{73-75}\) These types of capillaries could not be re-used, required time-consuming preparation of new capillaries, and limited the reproducibility of the method. Demonstrations reported by Stover\(^{71,76}\) or Wang\(^{67,70}\) detected \((P_i)_n\) by indirect UV absorbance (chromophores added to the running buffer included chromate or pyromelltic acid). These demonstrations did not however identify peaks for \((P_i)_n\) beyond \( P_3 \), nor did they quantify resolved \((P_i)_n\).

**Development of a CGE Method Exploiting Solutions of PDMA.** We used capillaries filled with solutions of PDMA to resolve \((P_i)_n\); UV-absorbance of terephthalate added to the running buffer enabled the indirect detection of \((P_i)_n\). The method we developed was convenient and rapid (analysis is complete within 20 minutes). Regenerating gel-filled capillaries, by pumping in fresh solutions of PDMA (after 5 runs of 20 minute duration), helped to ensure the reproducibility of the separation media and
results of analysis. Analysis of peak areas for (P)\textsubscript{n} and internal standards, determined by indirect UV-absorbance, allowed us to demonstrate the quantitative analysis of resolved (P)\textsubscript{n}. Development of the method involved optimizing several components (i.e., composition of the running buffer, sample preparation) described in the following section.

**EXPERIMENTAL DESIGN**

**Analytical Separation of (P)\textsubscript{n}**

**CZE – Separation of (P)\textsubscript{n} in Free Solution.** Results of CZE experiments were essential for guiding the development of a CGE technique. Although the resolution of mixtures of (P)\textsubscript{n} in free solution is poor, CZE experiments are easier to run than CGE experiments, and allowed us to i) explore the use of different chromophores for indirect detection, and identify terephthalate (TP\textsuperscript{2}) as an optimal choice; ii) measure \( \mu \) in free solution for standards of (P)\textsubscript{n}, and determine the influence of pH (in ranges of 6.8 - 7.1 and 8.4 - 8.7) and net electrostatic charge on the resolution of (P)\textsubscript{n}; iii) optimize the composition of the running buffer. Table 1 lists literature values of pK\textsubscript{a}. Analysis by CZE required the migration of (P)\textsubscript{n} to the anode and the use of capillaries with suppressed electro-osmotic flow. Capillaries covalently modified with a copolymer of \( N,N\)-dimethylacrylamide and 3-methacryloxy-propyltrimethoxysilane\textsuperscript{27} had electro-osmotic flow with mobilities < 0.1 cm\textsuperscript{2} kV\textsuperscript{-1} min\textsuperscript{-1}. 
Table 1. Values of pKa of Inorganic Polyphosphates.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>orthophosphate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.15, 7.20, 12.35</td>
</tr>
<tr>
<td>P2</td>
<td>pyrophosphate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8, 2.2, 6.7, 9.4</td>
</tr>
<tr>
<td>P3</td>
<td>tri(poly)phosphate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5, 1.0, 2.4, 6.5, 9.4</td>
</tr>
<tr>
<td>cyclo-P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>trimetaphosphate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(P&lt;sub&gt;n&lt;/sub&gt;)</td>
<td>polyphosphate&lt;sup&gt;d&lt;/sup&gt;</td>
<td>~1-2, 7.2-8.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values at infinite dilution and 25 °C, taken from Ref<sup>78</sup>.  
<sup>b</sup> Values at infinite dilution and 25 °C, taken from Ref<sup>79</sup>.  
<sup>c</sup> Results of titration of cyclo-P<sub>3</sub> cannot distinguish the pKa of the three ionizable groups of cyclo-P<sub>3</sub>; each of the three groups has a value of pKa of approximately 2.05.  
<sup>d</sup> Values taken from Ref<sup>9</sup>; ranges are inferred from titration curves for long-chain polyphosphates, and describe the strongly acidic hydrogen at each residue of phosphate (pKa of 1-2) and two weakly acidic hydrogen at the ends of the chain (pKa of 7.2-8.2).
CGE – Resolution of \((P_i)_n\) in Order of \(n\). To achieve a combination of speed, high resolution, and convenience for the analysis of mixtures of \((P_i)_n\), we ultimately decided to use electrophoresis through a solution of polymer. Aqueous solutions of PDMA (average molecular weight 57 kDa, 9.1% w/v) served as the sieving medium in CGE experiments. The main advantage of using a solution of PDMA is its low viscosity (< 100 cP), compared to that of other water-soluble polymers (of comparable molecular weight) applied in CGE, such as polyacrylamide\(^{80-81}\), polyvinylpyrrolidone\(^{82}\), hydroxypropyl cellulose\(^{83}\), polyethylene glycol\(^{84-85}\). This characteristic of PDMA allowed us to prepare gel-filled capillaries within 10 minutes by pumping solutions of PDMA into capillaries at easily obtained pressures (30 psi with ultra-high purity \(N_2\)). Other research groups have previously demonstrated the use of PDMA in CGE for the analysis of DNA.\(^{74,86-90}\)

Detection of \((P_i)_n\) by Indirect UV Absorbance. Species of \((P_i)_n\) cannot be detected by absorbance at wavelengths above 200 nm.\(^{91}\) Efforts to derivatize \((P_i)_n\) chemically with chromophores have not been successful.\(^{92-93}\) The use of electrophoresis to separate \((P_i)_n\) gave us the ability to detect anions (without chromophores) by indirect UV absorbance. We used the anionic chromophore terephthalate (\(TP^{2-}\)) for indirect detection, by adding terephthalic acid to solutions of running buffer (pH > 6.8; composition and properties of the buffer discussed below). \(TP^{2-}\) carries current throughout the capillary during electrophoresis. Migration of \(TP^{2-}\) by the detector produces a steady-state signal in UV absorbance, and generates the baseline. Sample zones containing \((P_i)_n\) migrate towards the anode and separate on the basis of mobility. Within a sample zone, the current is carried, at least in part, by \((P_i)_n\) rather than \(TP^{2-}\). Zones of separated \((P_i)_n\) can thus be detected by a decrease in UV absorbance by \(TP^{2-}\) from the baseline. The current along
the capillary – through zones of running buffer and through zones containing analyte – is constant and determined by the composition of the running buffer (i.e., concentration and mobility of the buffer ions)\textsuperscript{94}; further discussion of sample zones and indirect detection is available in the Supporting Information.

Terephthalate, TP\textsuperscript{2−}, absorbs at $\lambda = 254$ nm ($\epsilon = 8.2 \cdot 10^3$ M\textsuperscript{−1} cm\textsuperscript{−1}) and has mobility ($\mu \sim 28$ cm\textsuperscript{2} kV\textsuperscript{−1} min\textsuperscript{−1}) close to that of (P\textsubscript{i}n\textsubscript{26-34} cm\textsuperscript{2} kV\textsuperscript{−1} min\textsuperscript{−1} in free solution, pH = 7.0). Similar values of $\mu$ for TP\textsuperscript{2−} and (P\textsubscript{i}n are important for limiting the asymmetry and broadening of peaks, caused by dispersion in electromigration and de-stacking.\textsuperscript{94} The sensitivity of detection of different (P\textsubscript{i}n depends primarily on the extinction coefficient of TP\textsuperscript{2−}, and the mobilities of TP\textsuperscript{2−} and (P\textsubscript{i}n (quantities that are known or measured in CE); the estimated limit of detection of residues of P\textsubscript{i} is $\sim 0.2$ $\mu$M. The relationship between the area of an observed peak and the amount of (P\textsubscript{i}n analyzed is discussed in the Results section. Comparison of peak areas allowed us to determine the relative quantities of different species of (P\textsubscript{i}n in a mixture. Methods of detection used in other analytical techniques, such as conductivity (in LC) or staining by TBO (in PAGE), rely on properties of (P\textsubscript{i}n that change with $n$. Measuring the amount of a specific (P\textsubscript{i}n in mixtures by these methods requires additional information about the quantitative response factors (e.g., conductivity of as a function of $n$).

**Ions in the Running Buffer.** The running buffer, by design, contains only one type of anion, TP\textsuperscript{2−}. This design avoids complications in the indirect detection of (P\textsubscript{i}n by the absorbance of TP\textsuperscript{2−}. Anions in addition to TP\textsuperscript{2−} in the running buffer lead to system zones (that show up as negative peaks in indirect UV absorbance), artifacts in peak shape, and complications in the analysis of peak areas determined by indirect UV absorbance.\textsuperscript{94-98}
We therefore used buffers consisting only of bis-tris ($pK_a = 6.46$) or tris ($pK_a = 8.06$) and terephthalic acid ($pK_a = 3.51, 4.82$), prepared by adding terephthalic acid to solutions of bis-tris or tris (18.0 - 24.0 mM). The resulting buffers contained $TP^2\text{−}$ (3.0 mM), cationic protonated amine (6.0 mM bis-tris-H$^+$ or tris-H$^+$), and free amine (12.0 - 18.0 mM of bis-tris or tris).

**pH.** Running buffers had pH in the range of 6.8 – 7.2 (bis-tris) or 8.4 – 8.6 (tris). These buffers allowed us to test both ranges of pH on the resolution of $(P_i)_n$ in CZE and CGE. These values of pH are well beyond the $pK_a$ of the first ionizable group of phosphate residues (~2) but are near the $pK_a$ of the second ionizable groups of the terminal residues of phosphate of $(P_i)_n$ (~6.3 - 7.2) (see Table 1). Values of mobility for $(P_i)_n$ are sensitive to the pH of the running buffer, particularly for $n < 5$.

**Addition of Polyethylene Glycol (PEG) to Reservoirs of Running Buffer.** CGE experiments, unlike CZE experiments, involve discontinuous electrophoresis. Capillaries in CGE are filled a solution of entangled polymer, PDMA; the open ends of the capillary are immersed into reservoirs of running buffer that do not contain PDMA. Solutions of PDMA are more dense (~1.06 g/mL) than solutions of running buffer that do not contain PDMA (1.01 g/mL). Solutions of PDMA leaked out of the capillary into the buffer reservoirs, by gravity. The reproducibility of this experiment was therefore poor; the current decreased by > 10% within two hours, and retention times increased by > 5% in subsequent runs.

The solution to this problem was to add polyethylene glycol (PEG) to the reservoirs of running buffer, generating solutions isodense to the gel inside the capillary. Increasing the density of the solutions in the reservoirs by the adding glycerol or D$_2$O did not
improve the stability of the experiment. PEG is water-soluble and uncharged; it does not migrate during electrophoresis. CGE experiments using reservoirs of running buffer with 9.0% PEG (w/v) showed stable currents and improved run-to-run reproducibility. This procedure was essential for maintaining a constant medium for separation; we routinely collected data for 120 minutes of electrophoresis for each preparation of a filled capillary (enough for 5-6 typical analyses of (P$_i$)$_n$ samples).\textsuperscript{99}

**Loading Samples by Electrokinetic Injection.** In an applied electrical field, anions in the sample migrated towards the anode and entered the capillary. Electrokinetic injections transferred only anions, rather than entire plugs of solution, into the capillary. This procedure avoided creation of discontinuities within the gel-filled capillary, and improved the reproducibility of the separation media. Injections of (P$_i$)$_n$ by an electrical field can also pre-concentrate ions by isotachophoresis, enabling the analysis of samples that are dilute in (P$_i$)$_n$ (\textasciitilde\(\mu\)M).\textsuperscript{100-103} The total number of ions injected is determined by the electrical field, conductivity of the running buffer, and duration of the injection (and to a lesser extent, the composition of the sample; further discussion in the Supporting Information). Typical injections (175 V cm$^{-1}$ for 2.0 s) corresponded to the loading of \textasciitilde0.1 nmol of charge. The amount of each ion injected depends on both the concentration and mobility of the ion, as well as the concentration and mobility of all other ions in the sample. Our quantitative treatment of the data accounts for the bias introduced by electrokinetic injection (discussed in the Results section, and in further detail in the Supporting Information).\textsuperscript{104-105}

**Sample Preparation.** The procedure described above results in the injection and detection of all anions in a sample, not just (P$_i$)$_n$.\textsuperscript{106} The analysis of (P$_i$)$_n$ by indirect UV
detection works best on samples that are free of salts besides \((P_i)_n\). The following steps were used to prepare samples of \((P_i)_n\) that are free of additional ions (that originated from either synthesis or preparative separation): i) adsorption to anion-exchange resin; ii) elution of anions other than \((P_i)_n\) (e.g., \(\text{Cl}^-\)) with 0.1 M \(\text{Na}_2\text{CO}_3\); iii) elution of \((P_i)_n\) with concentrated 2.0 M \(\text{NH}_4\text{HCO}_3\); iv) removal of \(\text{NH}_4\text{HCO}_3\) under vacuum.\(^{107}\)

**Analytes.**

We optimized the components of the analytical method by testing the ability to resolve mixtures of \((P_i)_n\) of known chain length. Samples used to demonstrate the method consisted of purified samples of \((P_i)_n\) \((n \leq 10)\) that served as analytical standards, and mixtures of \((P_i)_n\) with \((1 < n < 100)\).

**Authentic standards of \((P_i)_n\) \((n = 4 - 10)\).** Commercially available oligophosphates of a single chain length are limited to the salts of \(P_1\), \(P_2\), \(P_3\), and cyclo-\(P_3\). Preparative-scale separation of mixtures, by ion-exchange chromatography, generated samples of isolated, oligomeric \((P_i)_n\), \(n = 4 - 10\). By analyzing samples consisting of these standards added to mixtures of \((P_i)_n\), we identified peaks that represented \((P_i)_n\) with \(n = 1 - 10\).

**Mixtures of \((P_i)_n\).** We demonstrated the resolution of the method by analyzing commercially available samples of higher \((P_i)_n\). Samples covering a range of average length \((\bar{n})\) were used to test the method: 117% polyphosphoric acid, \(\bar{n} = 17\), \(\bar{n} = 21\), \(\bar{n} = 48\), \(\bar{n} = 65\). In addition, we prepared samples of \((P_i)_n\) generated by the dehydration of \(\text{NH}_4\text{H}_2\text{PO}_4\) in mixtures with urea, using conditions reported by Orgel et.al., which were presumed to be plausible in prebiotic chemistry.\(^{4,23}\)
Internal standard(s). We added an internal standard, K\(^+\)CH\(_3\)SO\(_3\)^\(-\), to each sample prior to analysis by CE. The peak observed for the internal standard allowed us to: i) monitor the reproducibility of the method; ii) define the mobilities of species of \((P_i)_n\) relative to that of a standard (CH\(_3\)SO\(_3\)^\(-\)) (\(\mu_{P, rel}\) in Eq. 6); iii) compare the integrated area of the peak for the internal standard to the areas for analyte peaks, and to calibrate signal intensities to the concentrations of resolved \((P_i)_n\) in the sample.

\[
\mu_{P, rel} = \mu_p - \mu_{CH_3SO_3^-} = \frac{L_1 L_2}{V} \left( \frac{1}{t_{CH_3SO_3^-}} - \frac{1}{t_p} \right)
\]

In Eq. 6, \(t_{CH_3SO_3^-}\) and \(t_p\) are the retention times for CH\(_3\)SO\(_3\)^\(-\) and \((P_i)_n\); \(V\) is the voltage, \(L_1\) is the length of the capillary, and \(L_2\) is the length between the inlet and detector. The electrophoretic mobility of CH\(_3\)SO\(_3\)^\(-\) in free solution was near that of \((P_i)_n\), but peaks for CH\(_3\)SO\(_3\)^\(-\) and \((P_i)_n\) did not overlap (\(\mu_{CH_3SO_3^-} = 26.7 \text{ cm}^2 \text{ kV}^{-1} \text{ min}^{-1}\)). In experiments that required additional internal standards with different values of mobility, we also used Cl\(^-\), CF\(_3\)CO\(_2\)^\(-\), and CH\(_3\)-C\(_6\)H\(_4\)-SO\(_3\)^\(-\).

RESULTS & DISCUSSION

Resolution and Detection of P\(_1\), P\(_2\), P\(_3\), and cyclo-P\(_3\) by CZE. Analysis of mixtures of P\(_1\), P\(_2\), P\(_3\), and cyclo-P\(_3\) by CZE, with indirect UV-absorbance, demonstrated the resolution of lower oligophosphates. Using coated capillaries and running buffer composed of 3.0 mM TP\(^2\)^\(-\) and 18 - 24.0 mM bis-tris or tris, we obtained the data in Fig. 1. Samples were loaded by electrokinetic injection at the cathode (10 kV for 2.0 s) and
separated by electrophoresis towards the anode (14.6 kV). The steady-state absorbance signal for TP$_2^-$ at 254 nm enabled the indirect detection of anions from the sample. Analysis of pure samples of P$_1$, P$_2$, P$_3$, and cyclo-P$_3$ led to the identification of the peaks (labels above traces). Coated capillaries (100 µm in inner diameter, 57 cm in length, and 50 cm between inlet and detector) were prepared by reaction of the surface with a copolymer of N,N-dimethylacrylamide and 3-methacryloxy-propyltrimethoxysilane; experiments with coated capillaries used over the course of several months, for more than 100 runs, showed no observable change in retention time for analytical standards.

Peaks in Trace A show values of mobility in the order of cyclo-P$_3$ > P$_3$ > P$_2$ > P$_1$. This order is consistent with the number of ionizable groups, values of pK$_a$ and structures of these (P$_i$)$_n$ (i.e., the radius of cyclo-P$_3$ is constrained in a way that P$_3$ is not). Trace B shows that P$_1$ migrates more rapidly in a buffer with higher pH (8.4 vs. 6.8). The increase in mobility of P$_1$ is consistent with an increase in negative charge determined by the extent of dissociation of the second acidic hydrogen of P$_1$ (pK$_a$ = 7.2). CZE does not resolve P$_3$ and P$_2$ at pH = 8.4. The reason for the migration of P$_3$ and P$_2$ that is less rapid at pH = 8.4 than at pH = 6.8 is not clear; possible reasons include i) trace amounts of metal ion affecting the mobilities of P$_2$, P$_3$, and higher (P$_i$)$_n$, or ii) a buffer-specific effect$^{108-109}$, e.g., association of P$_2$ and P$_3$ to tris-H$^+$ with greater affinity than association to bis-tris-H$^+$. 

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Figure 1. Resolution of P₁, P₂, P₃, and cyclo-P₃ by CZE. Traces A and B analyze mixtures of P₁, P₂, P₃, and cyclo-P₃ in free solution, using running buffers with pH = 6.9 and pH = 8.4 at 25 °C. Traces C and D analyze mixtures of inorganic ions (Cl⁻, CH₃SO₃⁻, CF₃CO₂⁻, CH₃-C₆H₄SO₃⁻).
For anions with $pK_a$ below the pH of the running buffer by more than three units (Cl$^-$, CH$_3$SO$_3^-$, CF$_3$CO$_2^-$, CH$_3$C$_6$H$_4$SO$_3^-$), peaks show mobilities that do not change with pH, and do not overlap with peaks for (P$_i)_n$ (traces C and D). These characteristics are ideal for their use as internal standards. We used CH$_3$SO$_3^-$ as an internal standard for the remainder of our experiments.

**Resolution of mixtures of (P$_i)_n$ by CGE.** The resolution of broad distributions of (P$_i)_n$ required the use of a sieving gel. Best results were obtained with capillaries filled with solutions composed of 9.1% PDMA (w/v; average molecular weight 58.9 kDa) and running buffer (24.0 mM tris, 3.0 mM terephthalic acid, pH = 8.4). We filled capillaries (100 µm internal diameter and 57 cm in length) by pumping in solutions of PDMA with positive pressure (30 psi of ultra-high purity N$_2$ applied to the inlet) for 20 minutes. During electrophoresis, the ends of the capillary were immersed into reservoirs of running buffer containing 9% PEG (w/v; average molecular weight of 1.5 kDa); solutions in buffer reservoirs were isodense with the solution inside the capillary.

The upper trace in **Figure 2A** shows the separation of (P$_i)_n$ from a commercially available mixture of sodium polyphosphate (reported chain length of $\bar{n} \sim 17$). The sample had a concentration of 19.0 mM (in phosphate residues) and contained P$_3$ (2.0 mM) and CH$_3$SO$_3^-$ (2.0 mM), added to serve as internal standards. The lower trace in Fig. 2A shows the analysis of the same sample by CZE, for comparison, using a running buffer with the same composition used in CGE.

We identified peaks for P$_1$, P$_2$, and P$_3$ (marked with a dotted line in Fig. 2B) by comparing the traces in Fig. 2 to those collected for samples containing standards added to the mixture. In CGE experiments, mobilities are lower for (P$_i)_n$ having $n > 3$ than for
Figure 2. Resolution of \((P_i)_n\) by CGE. A) Analysis of sodium polyphosphate (average chain length 17) in capillaries filled with 9.1% PDMA (w/v) gel (upper trace) or running buffer alone (24.0 mM tris, 3.0 mM terephthalic acid, pH = 8.4, 25 °C) (lower trace). Electrophoresis was performed by applying 14.6 kV across capillaries having a length of 57 cm (50 cm from the inlet to detector). B), C) Expanded views of the upper and lower traces in 2A), respectively.
2B)

![Graph showing peaks labeled $P_2$, $P_3$, $CH_3SO_3^-$ and n = \(5, 10, 15, 20, 25, 30, 35\).](image)

Time / min

2C)

![Graph showing peaks labeled $P_2$, $CH_3SO_3^-$.](image)

Time / min
In CZE, the mobilities of all \((P_i)_n\) are greater than that of \(\text{CH}_3\text{SO}_3^-\). The contrast between CGE and CZE, in the order of mobilities, indicates size-sieving during CGE, and the separation of \((P_i)_n\) in order of \(n\). The series of peaks detected by CGE (shown in 2B) suggests species as large as \(~P_{35}\) in the sample.

**Preparation of \((P_i)_n, n = 4 - 10.** Assigning specific \((P_i)_n\) to peaks in broad distributions of \((P_i)_n\), such as the one in Fig. 2A, required oligophosphate standards that were not commercially available. Neutralizing samples of 117% polyphosphoric acid\(^{110}\) with NaOH\(_{(aq)}\) generated mixtures of \((P_i)_n\) with \(n \sim 1 - 15\). The mixture was separated on an anion-exchange chromatography column (Cl\(^-\) form) by elution with a gradient of KCl\(_{(aq)}\). A second application of anion-exchange chromatography removed KCl from fractions containing \((P_i)_n\) (HCO\(_3^-\) form; elution with NH\(_4\)HCO\(_3\)). Removal of NH\(_4\)HCO\(_3\) by vacuum generated oligophosphate standards as the ammonium salt.

Characterization of purified \((P_i)_n, n = 4 - 10,\) by CZE shows one major peak for each sample (Fig. 3). Smaller peaks adjacent to the major peak and a very small peak at the migration time of P\(_1\) (top trace in Fig. 3 for reference) suggest small amounts of the species \((P_i)_{(n + 1)}\) or \((P_i)_{(n - 1)}\) and very little P\(_1\). The relatively clean traces suggest that the conditions used in the preparation of samples do not cause the hydrolysis of \((P_i)_n\) or equilibration in chain length. Characterization of oligophosphate standards by \(^{31}\text{P} \text{NMR}\) is available in the Supporting Information.
Figure 3. Standards of \((P_n)_n\), \(n = 1 – 10\). Data collected by CZE show the analysis of i) commercially available \(P_1\), \(P_2\), \(P_3\), and \(cyclo-P_3\), with \(CH_3SO_3^-\) added as an internal standard and ii) \(P_4 – P_{10}\) purified by anion exchange chromatography (no internal standard added). The CZE protocol was the same as the one used to collect the data in Fig. 1A.
Identification of Peaks for $n = 3 - 10$ in Mixtures of $(P_i)_n$. Analysis of mixtures of $(P_i)_n$ (19.0 mM in phosphate residues; $\bar{n} \sim 17$) and added $P_3$, $P_4$, $P_6$, $P_8$, and $P_{10}$ (2.0 mM) by CGE generated the data in Fig. 4. Small changes in migration times from run to run prevent the direct comparison of raw data analyzed in units of time. The $x$-axis of traces in Fig. 4 are in units of mobility relative to $\text{CH}_3\text{SO}_3^-$ $\mu_{\text{p,rel}}$ (Eq. 6). Traces plotted in units of mobility show the alignment of peaks from run to run. Comparison of traces allowed us to assign specific $(P_i)_n$ to peaks with increased area (dotted lines in Fig. 4A). The dotted line for $n = 20$ in Fig. 4B is based on the reasonable assumption that the peaks continue in order of $n$.\textsuperscript{111}

Mixtures of $(P_i)_{n}$ with $n$ up to 100. Electropherograms in Fig. 5 characterize commercially available mixtures of $(P_i)_n$ having different distributions in chain length. Samples analyzed in A-D (source given to the right) are in order of increasing average chain length, $\bar{n}$. The results show that peaks with $n > 70$ can be resolved in a single run; larger $(P_i)_n$ may be resolvable using gels with lower concentrations of PDMA.

Shapes of Peaks. The asymmetric peaks for $(P_i)_n$ observed in both CZE and CGE experiments are typical for electropherograms collected by indirect UV absorbance. Peaks for analytes with mobilities higher than TP$^2-$ have a sloping front, while peaks for analytes with mobilities lower than TP$^2-$ have a sloping tail. These shapes are the results of dispersion by electromigration caused by i) differences in mobility between analytes and TP$^2-$, and ii) non-uniform electric fields inside sample zones. Discussion of these effects is available in Ref. 92; scheme 1 in the Supporting Information illustrates the formation of asymmetric peaks.
**Figure 4. Identification of P₃ – P₁₀ in mixtures of (Pᵦₙ by CGE.** Traces are for the analysis of mixtures of (Pᵦₙ (19 mM in total phosphate, pH ~ 8) and added standards for (Pᵦₙ (2 mM). Mixtures were resolved by CGE at 14.6 kV using capillaries filled with 9.1 % PDMA (w/v) and running buffer (24.0 mM tris, 3.0 mM TP²⁻, pH = 8.4). Traces are shown in units of mobility relative to the internal standard CH₃SO₃⁻ (Eq. 6). Dotted lines mark peaks for species identified by added standards. Traces for samples with added P₇, or P₉, are available in the Supporting Information.
Figure 5. Resolution of commercially available mixtures of \((P_i)_n\). Samples consisting of commercially available mixtures of polyphosphate glass (20 mM in total phosphate, Na\(^+\) salt) and CH\(_3\)SO\(_3^-\) (2 mM) were analyzed by CGE. After electrokinetic injection (4.0 s at 10 kV), \((P_i)_n\) were separated by electrophoresis at 14.6 kV in coated capillaries (57 cm in length, 50 cm between inlet and detector) filled with solutions of 9.1% PDMA (w/v) and running buffer (24.0 mM tris, 3.0 mM TP\(^{2-}\), pH = 8.4). Traces showing indirect UV absorbance are in units of mobility relative to the internal standard, CH\(_3\)SO\(_3^-\), to facilitate run-to-run comparison. Peaks in trace C, for \(n \sim 10 - 40\), appear less sharp than the peaks in other runs. The reason for this difference is unclear; one possibility is that the peaks are affected by irregularities in pH within capillaries during separation, caused by a small mismatch in pH between samples (~ 8) and the running buffer (pH = 8.4).
Inset to Figure 5:

![Graph showing mobility relative to CH$_3$SO$_3^-$](image)

- sample analyzed
  - A) 117% polyphosphoric acid
  - B) polyphosphate glass 25
  - C) polyphosphate glass 45
  - D) polyphosphate glass 65

negative absorbance (254 nm)

mobility relative to CH$_3$SO$_3^-$ (cm$^2$ kV$^{-1}$ min$^{-1}$)
Quantitative analysis of \((P_i)_n\).

**Areas of Peaks.** Three contributions determine the area of a peak: i) the response of 
\([TP^2\text{]}\) to \((P_i)_n\); ii) the amount of \((P_i)_n\) transferred from the sample to the capillary by 
electrokinetic injection; iii) residence time of the analyte passing the detector. Analysis 
of areas of peaks for resolved \((P_i)_n\) and \(\text{CH}_3\text{SO}_3^-\) can quantify the amount of specific \((P_i)_n\) in a mixture. To account for the effect of iii), areas of peaks detected by CZE or CGE are 
adjusted by multiplying them by the factor \((1 / t_i)\), where \(t_i\) is the retention time.\(^{112}\) The 
ratio of adjusted areas of peaks for analyte \(i\) and \(\text{CH}_3\text{SO}_3^-\) \((A_i\text{ and } A_{\text{CH}_3\text{SO}_3^-})\) is related to 
the concentration of \(i\) and \(\text{CH}_3\text{SO}_3^-\) in the sample by Eq. 7:

\[
\frac{A_i}{A_{\text{CH}_3\text{SO}_3^-}} = \frac{[i]_S}{[\text{CH}_3\text{SO}_3^-]} \left(\frac{z_i}{z_{\text{CH}_3\text{SO}_3^-}}\right) \left(\frac{\mu_{\text{C}^+} + \mu_i}{\mu_{\text{C}^+} + \mu_{\text{CH}_3\text{SO}_3^-}}\right)
\]  

\text{(7)}

In Eq. 7, \(z_i\) and \(z_{\text{CH}_3\text{SO}_3^-}\) are the electrostatic charge of \(i\) and \(\text{CH}_3\text{SO}_3^-\); \(\mu_{\text{CH}_3\text{SO}_3^-}\) and \(\mu_i\) are 
the mobilities of \(i\) and \(\text{CH}_3\text{SO}_3^-\); \(\mu_{\text{C}^+}\) is the mobility of the cation in the sample zone (bis-
tris-\(\text{H}^+\) or tris-\(\text{H}^+\)). The Supporting Information contains our derivation of Eq. 7, based 
on explanation of quantitative aspects of electrokinetic injections and indirect UV 
absorbance.

Equation 7 reveals the advantage of analyzing electropherograms by comparing peaks 
for \((P_i)_n\) and for the added standard. The ratio \((A_i / A_{\text{CH}_3\text{SO}_3^-})\) depends on the ratio of the 
concentrations \(([i]_S / [\text{CH}_3\text{SO}_3^-])\) but does not depend on the concentration of other ions 
in the sample, or the voltage and duration of electrokinetic injection.
Calibration of peak areas to concentrations of $P_1$, $P_2$, $P_3$ and cyclo-$P_3$. Analysis of samples of containing $P_1$, $P_2$, $P_3$, or cyclo-$P_3$, and added CH$_3$SO$_3^-$ by CZE demonstrated the relationship between peak areas and concentrations. Fig. 6 shows values of $(A_i / A_{CH_3SO_3^-})$ determined from peaks in the analysis of cyclo-$P_3$ and CH$_3$SO$_3^-$. Samples had ratios of cyclo-$P_3$ to CH$_3$SO$_3^-$ that spanned a factor of $10^4$. The points are consistent with a linear dependence of $(A_i / A_{CH_3SO_3^-})$ on $([i]S / [CH_3SO_3^-])$. The dotted line with slope $m$ was obtained by fitting to the points with adjusted weighting. Data collected for the quantitative analysis of $P_1$, $P_2$, and $P_3$ are available in the Supporting Information.

The peak areas of cyclo-$P_3$ and CH$_3$SO$_3^-$ are consistent with the quantitative relationship predicted by Eq. 7. A value of 0.99 estimated for the slope ($m$) in Fig. 6 is consistent with the complete ionization of all three acidic groups of cyclo-$P_3$ at pH = 6.8 ($pK_a ~ 2.05$ for all three groups). This calibration of ratios of peak areas of cyclo-$P_3$ to CH$_3$SO$_3^-$ provides a way to measure the amount of cyclo-$P_3$ in mixtures. Calibration for other $(P_i)_n$ should enable the quantitative estimation of several $(P_i)_n$ in mixtures.

Analysis of $(P_i)_n$ Generated by Dehydrating $P_i$.

CGE provides a useful method for exploring reactions that may have generated $(P_i)_n$ on prebiotic earth. CGE allowed us to characterize samples prepared by dehydrating $P_i$ in mixtures with urea, using conditions originally reported by Orgel. The mechanism of dehydration has not been established. Urea is however essential; heating NH$_4$H$_2$PO$_4$ in the absence of urea only generates a small amount of $P_2$.

Evaporating solutions of NH$_4$H$_2$PO$_4$ (0.2 mmol) and urea (20.0 mmol) at temperatures $\geq 125$ °C generated $(P_i)_n$. After 48 hours of heating mixtures to either
Figure 6. Quantitative Calibration of cyclo-P₃. Data in the plot are for \( \frac{A_{cyclo-P_3}}{A_{CH_3SO_3^-}} \) determined in the analysis of samples of cyclo-P₃ and CH₃SO₃⁻ by CZE, using coated capillaries (57 cm in length, 50 cm between inlet and detector) filled with running buffer (18.0 mM bis-tris, 3.0 mM TP²⁻, pH = 6.9). Points for \( \left( \frac{[cyclo-P_3]}{[CH_3SO_3^-]} \right) = 0.1, 1.0, \text{ and } 10.0 \) are the average taken from eight experiments. Standard deviations for \( \left( \frac{A_{cyclo-P_3}}{A_{CH_3SO_3^-}} \right) \) are < 5% of the measured values; error bars are not visible in plots with a logarithmic scale. Points at other values of \( \left( \frac{[cyclo-P_3]}{[CH_3SO_3^-]} \right) \) are from one or two trials. The slope \( m \) is for the line determined by fitting the data with adjusted weighting.¹¹³
125 °C or 140 °C, we prepared samples for CGE by cooling the mixtures to 25 °C, adding water (2.0 mL), and removing insoluble material by centrifugation. Analysis of the samples by CGE, shown in Fig. 7, showed the formation of \((P_i)_n\) with \(n > 40\), and distinguished the composition of mixtures generated at 125 °C and 140 °C. *Cyclo*-\(P_3\) is the most abundant species in mixtures prepared at 140 °C. In contrast, the distribution of \((P_i)_n\) prepared at 125 °C does not contain *cyclo*-\(P_3\), despite containing linear \((P_i)_n\) having \(n > 50\). The reason for the preferential formation of *cyclo*-\(P_3\) over linear \((P_i)_n\) at 140 °C is not clear; the synthesis of *cyclo*-\(P_3\) is however potentially important for prebiotic chemistry. Reactions of \((P_i)_n\) with -OH groups, leading to phosphorylated and polyphosphorylated organic compounds, likely depend on whether the \((P_i)_n\) are in cyclic or linear (chain) structures. Reactions of linear \((P_i)_n\) potentially transfer phosphate residues from either terminal or middle positions of the chain, while reactions of *cyclo*-\(P_3\) are ring-opening and can lead to triphosphate compounds that are analogous to ATP. The resolution and convenience of CGE will enable the broad survey of conditions for the synthesis of \((P_i)_n\) from \(P_i\), and for reactions of \((P_i)_n\) with -OH groups. The results should be helpful towards refining hypotheses for the importance of dehydrated phosphate in the chemical origins of life.

**CONCLUSION**

Previously reported methods using electrophoresis (slab gels or CGE) to analyze \((P_i)_n\) successfully resolved species on the basis of their size \((n)\). These methods provided a way to qualitatively characterize mixtures of \((P_i)_n\) at high resolution, but involved difficult and time-consuming experiments with limited reproducibility. The method we
Figure 7. Analysis of \((P_i)_n\) Generated under Possible Prebiotic Conditions. A)

Samples of \((P_i)_n\), generated by heating mixtures of \(\text{NH}_4\text{H}_2\text{PO}_4\) and urea, were 20.0 mM in total phosphate with pH \(~ 7\). Analysis by CGE used coated capillaries filled solutions of PDMA (9.1% w/v) and running buffer (3.0 mM TP\(^{2-}\) and 24.0 mM tris, pH = 8.4); the protocol for CGE was the same used to collect the data in Fig. 4. B) Close-up views of peaks observed in the range \(\mu = 3 – 9 \text{ cm}^2 \text{ kV}^{-1} \text{ min}^{-1}\). Peaks marked with a * have not been identified. The reason for the irregular shapes of peaks for \(n \sim 10 - 15\) in the analysis of \((P_i)_n\) synthesized at 125 °C is unclear, but is probably related to the preparation of this specific sample, and not the method; peaks for \(n \sim 5 - 20\) appear sharp in the analysis of other mixtures when the same procedure (Figs. 2 and 4). One possible reason for peak broadening in some parts of the trace, but not others, is non-uniform pH in the capillary during separation, and subsequent electrodispersion. Irregularities in pH may originate from either i) a mismatch in pH between running buffer and sample, or ii) bias favoring the injection of ions with negative charge and higher mobility, during electrokinetic injection, resulting in a pH for the sample plug that is different from the pH of the sample solution.
have demonstrated in this paper exploits the sieving properties of low-viscosity solutions of PDMA, and is capable of high resolution and quantitative analysis. The advantages of CGE using solutions of PDMA as the separation medium are convenient preparation, rapid analysis, and reproducibility (enabled by refilling capillaries with fresh solutions of gel). Our identification of P₁-P₁₀ and resolution up to P₇₀ validates a method that will be useful in studies of (Pᵢ)ᵣ relevant to prebiotic chemistry and biochemistry.

In addition to characterizing the composition of samples of (Pᵢ)ᵣ, the method we have developed is potentially useful for analyzing other anionic, oligomeric species without a sensitive chromophore (single-stranded DNA and RNA are easily detected by UV absorbance; kits of reagents and supplies for analyzing RNA and DNA are commercially available). Examples relevant to prebiotic chemistry are oligomers of phosphate condensed with organic compounds (e.g., structures with formula (PO₃⁻-RO)ᵣ or (P₂O₆²⁻-RO)ᵣ)). Examples of biological polymers include teichoic acid, hyaluronic acid, and sulfated polysaccharides such as heparin and chondroitin sulfate.

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SUPPORTING INFORMATION AVAILABLE.
Additional information as noted in the text, as well as information about experimental procedures and sources of chemicals, is available free of charge at http://pubs.acs.org.

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(99) We used PEG instead of PDMA in buffer reservoirs because commercial PDMA (~$100 per g) is much more expensive than PEG (~ $0.1 per g); each set of experiments requires ~ 1 g of polymer; PDMA can be obtained inexpensively however, by preparing it from N,N-dimethylacrylamide.
(110) The nomenclature (i.e., 117%) is based on comparing the ratio of phosphorus to oxygen in samples of dehydrated phosphate to the ratio of a standard solution of 85% phosphoric acid.
(111) The alignment is not perfect; small differences from run to run are possibly due to dispersion in electromigration, caused by differences between samples in pH or ionic strength.
(113) Values of the points span a factor of $10^4$. Errors are correlated with the independent variable (ratio of concentrations in the sample). A line fit to these points is determined mostly by the value and error for the highest ratio tested, $10^2$. We obtained a better fit by linear regression of log ($A_i / A_{CH_3SO_3^-}$) to log ([i]S / [CH$_3$SO$_3^-$]) with a slope constrained to a value of one. Fitting the equation log $y = \log m + \log x$ to the data provides a more accurate result for points that are separated by orders of magnitude than fitting to the equation $y = mx$ (the form of Eq. 7); $y$ is ($A_i / A_{CH_3SO_3^-}$), $x$ is ([i]S / [CH$_3$SO$_3^-$]), and $m$ is a proportionality constant obtained by fitting to the data.