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Analysis of Inorganic Polyphosphates by Capillary Gel Electrophoresis

Andrew Lee and George M. Whitesides*

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA

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 $(P_i)_n$ (e.g., gel electrophoresis, CGE using polyacrylamide-filled capillaries) are its resolution, convenience, and reproducibility; gel-filled capillaries are easily regenerated 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).

This paper describes a technique for analyzing mixtures of inorganic oligo- and polyphosphates, $(P_i)_n$, by capillary gel electrophoresis. Samples of condensed inorganic phosphate are typically mixtures of $(P_i)_n$ with different chain length, *n*. 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. Our primary motivation to develop a new method of analysis was to explore the synthesis and reactivity of $(P_i)_n$ relevant to the chemical origins of life (i.e., the prebiotic chemistry leading to self-replicating systems in a "pre-RNA" or "RNA world").[2−7]

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 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 $(P_i)_n$ in mixtures 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 cyclic and linear $(P_i)_n$ in order of their electrophoretic mobility. Addition of the chromophoric anion, terephthalate (1,4-$(CO_2^-)_2C_6H_4$, abbreviated as TP^{2-}), to the running buffer enabled the detection of separated $(P_i)_n$ by indirect UV absorbance.

We demonstrated the resolution of mixtures of $(P_i)_n$ (with *n* up to ~70) and the identification of components in mixtures with the use of authentic standards. The areas of peaks in electropherograms, determined 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 (>220 °C), we analyzed $(P_i)_n$ generated by dehydration reactions that might have occurred on the prebiotic earth and, thus, might have been involved in the chemical origins of life.[5,6,8]

MOTIVATION

$(P_i)_n$ and adenosine triphosphate (ATP) have an essential functional group in common: residues of dehydrated phosphate connected by phosphoanhydride bonds. $(P_i)_n$ is simpler in composition than ATP but, in principle, may provide the same chemical function (e.g., activation of −OH groups) and 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.[9−13] We

* To whom correspondence should be addressed. E-mail: gwhitesides@gmwgroup.harvard.edu.

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wished to explore the dehydration of P_i in detail and needed a method that was more convenient and reproducible than those reported so far for the resolution of mixtures of $(P_i)_n$ varying in chain length. The method we have developed should also be useful for investigating the biochemistry of $(P_i)_n$[9−11] and for developing $(P_i)_n$ as a reagent in chemical synthesis. 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).[14]

PREVIOUSLY REPORTED METHODS FOR SEPARATING MIXTURES OF $(P_I)_N$

Polyacrylamide gel electrophoresis (PAGE) can resolve mixtures of $(P_i)_n$, with $n \sim 2-450$. Analysis by PAGE involves gel electrophoresis (typical runs require ≥3 h) and the detection of $(P_i)_n$ by staining gels with the cationic dye toluidine blue O (TBO);[1,15,16] autoradiography can detect species of $(P_i)_n$ synthesized from ^{32}P-ATP.[17] 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 homogeneous and provide reproducible resolution; to run a gel requires several hours (for separation, staining, and destaining).

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 ∼50 peaks, in runs of less than 30 min;[18,19] this method requires HPLC instrumentation with a suppressed conductivity detector and an online KOH gradient generator (for minimizing the amount of CO_2/CO_3^{2-} adsorbed from the 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.

Other qualitative or semiquantitative methods used to analyze mixtures of $(P_i)_n$ include paper chromatography,[20,21] ^{31}P NMR,[22−27] ESI-MS,[28] and the analysis of terminal phosphate groups with phosphoglucokinase.[20]

CAPILLARY ELECTROPHORESIS OF $(P_I)_N$ AND DNA

Capillary electrophoresis separates and resolves analytes based on differences in electrophoretic mobility. Since both the amount of negative charge and hydrodynamic drag of $(P_i)_n$ increase with n, in a way similar to that of single-stranded DNA, we expected poor resolution in the analysis of $(P_i)_n$ in free-solution capillary electrophoresis (i.e., CZE).[29] Capillaries filled with a sieving matrix, however, are capable of resolving DNA in order of chain length.[30−32] The use of replaceable solutions of entangled polymer (such as linear acrylamide or PDMA) in CGE enabled the automated and massively parallel analysis of samples of DNA and was essential to the completion of the Human Genome Project.[33,34]

Previous reports of methods for analyzing $(P_i)_n$ by CGE used capillaries coated and filled with linear polyacrylamide (capillaries were prepared by filling capillaries with aqueous acrylamide and polymerizing in situ).[35,36] We, and others, found that capillaries prepared this way had short lifetimes;[37−40] these capillaries could not be reused, required time-consuming preparation of new capillaries, and limited the reproducibility of the method. Stover[36,41] and Wang[29,35] detected $(P_i)_n$ by indirect UV absorbance in CGE experiments (chromate or pyromelltic acid were the chromophores added to the running buffer). These demonstrations did not, however, identify peaks for $(P_i)_n$ beyond P_3 nor did they quantify resolved $(P_i)_n$.

EXPERIMENTAL DESIGN

CZE: Separation of $(P_i)_n$ in Free Solution. We used the results of CZE experiments to guide the development of a CGE technique. Although the resolution of mixtures of $(P_i)_n$ in free solution is poor, CZE experiments are easier to run than CGE experiments and allowed us to (i) test different chromophores for indirect detection and identify terephthalate (TP^{2-}) as an optimal choice; (ii) measure μ for analytical standards of $(P_i)_n$ in free solution and determine the influence of pH and net electrostatic charge on the mobility of $(P_i)_n$ (pH in the ranges of 6.8−7.1 and 8.4−8.7); (iii) optimize the composition of the running buffer. Table 1 gives literature values of pK_a for $(P_i)_n$. Analysis by CZE required the migration of $(P_i)_n$ to the anode and the use of capillaries with suppressed electro-osmotic flow. Capillaries covalently modified by reaction of the fused silica surface with a copolymer of *N*,*N*-dimethylacrylamide and 3-methacryloxy-propyltrimethoxysilane[42] had an electro-osmotic flow of <0.1 $cm^2\ kV^{-1}\ min^{-1}$.

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Table 1. Values of pK_a of Inorganic Polyphosphates

abbreviation	name	pK_a
P_1	orthophosphate[a]	2.15, 7.20, 12.35
P_2	pyrophosphate[a]	0.8, 2.2, 6.7, 9.4
P_3	tri(poly)phosphate[b]	0.5, 1.0, 2.4, 6.5, 9.4
cyclo-P_3	trimetaphosphate[a]	2.05[c]
$(P_i)_n$	polyphosphate[d]	~1−2, 7.2−8.2[d]

[a] Values at infinite dilution and 25 °C, taken from ref 57. [b] Values at infinite dilution and 25 °C, taken from ref 58. [c] Results of titration of *cyclo*-P_3 cannot distinguish the pK_a of the three ionizable groups of *cyclo*-P_3; each of the three groups has a value of pK_a of approximately 2.05. [d] Values taken from ref 14; ranges are inferred from titration curves for long chain polyphosphates and describe the strongly acidic hydrogen at each residue of phosphate (pK_a of 1 to 2) and two weakly acidic hydrogens at the ends of the chain (pK_a of 7.2−8.2).

CGE: Resolution of $(P_i)_n$ in Order of *n*. To achieve a combination of high resolution, speed, and convenience in the analysis of $(P_i)_n$, we used CGE with solutions of PDMA (average molecular weight of 57 kDa, 9.1% w/v) as the sieving medium.

A key advantage of the use of a solution of PDMA[39,43−47] is its low viscosity (<100 cP), compared to that of other water-soluble polymers applied in CGE, such as polyacrylamide,[48,49] polyvinylpyrrolidone,[50] hydroxypropyl cellulose,[51] and polyethylene glycol.[52,53] We prepared gel-filled capillaries by pumping solutions of PDMA into capillaries for 10 min at 30 psi. Regenerating gel-filled capillaries, by pumping in fresh solutions of PDMA (after 5 runs of 20 min duration each), ensured the reproducibility of the separation media.

Detection of $(P_i)_n$ by Indirect UV Absorbance. $(P_i)_n$ cannot be detected by absorbance at wavelengths above 200 nm.[54] Efforts to derivatize $(P_i)_n$ chemically with chromophores have not been successful.[55,56] Electrophoresis, however, provides the opportunity 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 of the buffer discussed below). TP^{2-} carries current in the capillary and migrates to the anode during electrophoresis. Migration of TP^{2-} by the detector produces a steady-state signal in UV absorbance, generating the baseline of electropherograms. Sample zones containing $(P_i)_n$ also migrate toward the anode. Within a sample zone, the current is carried in part by $(P_i)_n$, rather than TP^{2-}. Zones containing $(P_i)_n$ are detected by a decrease in UV absorbance, resulting from a decrease in the concentration of TP^{2-}.

The sensitivity of detection of $(P_i)_n$ depends primarily on the extinction coefficient of TP^{2-} and the mobilities of TP^{2-} and $(P_i)_n$. Terephthalate, TP^{2-}, absorbs at $\lambda = 254$ nm ($\varepsilon = 8.2 \cdot 10^3$ M^{-1} cm^{-1}) and has mobility ($\mu \sim 28$ cm^2 kV^{-1} min^{-1}) close to that of $(P_i)_n$ (26−34 cm^2 kV^{-1} min^{-1} in free solution, pH = 7.0). The similarity in μ for TP^{2-} and $(P_i)_n$ is important for limiting dispersion in electromigration and destacking, which result in the asymmetry and broadening of peaks.[59] The estimated limit of detection of residues of P_i is ~0.2 μM.

Ions in the Running Buffer. The running buffer, by design, contains only one type of anion: TP^{2-}. This characteristic avoids complications in the indirect detection of $(P_i)_n$ by the absorbance of TP^{2-}. Anions in addition to TP^{2-} would lead to system zones (that show up as negative peaks unrelated to $(P_i)_n$), artifacts in peak shape, and complications in the analysis of peak areas.[59−63] We, therefore, used buffers prepared by adding terephthalic acid to solutions of 18.0−24.0 mM bis−tris (pK_a = 6.46) or tris (pK_a = 8.06). The resulting buffers contained TP^{2-} (3.0 mM), 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 made with bis−tris or tris allowed us to compare the resolution of $(P_i)_n$ at two ranges of pH: 6.8−7.2 (bis−tris) and 8.4−8.7 (tris). 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) (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. In CGE, capillaries are filled with a solution of PDMA (density ~1.06 g/mL); the open ends of the capillary are immersed into reservoirs of running buffer that do not contain PDMA (~1.01 g/mL). As a result, solutions of PDMA leak out of the capillary into the buffer reservoirs, under gravity. The reproducibility of this experiment was, therefore, poor; the current decreased by >10% within 2 h, and retention times increased by >5% in each subsequent run.

The solution to this problem was to add polyethylene glycol (PEG) to the reservoirs of running buffer, generating solutions isodense with the solution inside the capillary. 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

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constant medium for separation; we routinely collected data for 120 min of electrophoresis for each preparation of a filled capillary (enough for 5 to 6 typical analyses of $(P_i)_n$).[64]

Loading Samples by Electrokinetic Injection. During electrokinetic injection, an applied field forces anions in the sample to enter the capillary and migrate toward the anode. Rather than transferring plugs of solution into the capillary, electrokinetic injection only transfers anions. This procedure avoids the creation of discontinuities in the capillary and improves the reproducibility of separation medium.

The total number of ions injected is determined by the electrical field, conductivity of the running buffer, duration of the injection, and to a smaller extent, the conductivity of the sample (further discussion in the Supporting Information). Typical injections (175 V cm^{-1} for 2.0 s) correspond to the loading of ~0.1 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 in sampling caused by electrokinetic injection (discussed in the Results and Discussion section and Supporting Information).[65,66]

Sample Preparation. The procedure described above results in the injection and detection of all anions in a sample, not just $(P_i)_n$.[67] Analysis of $(P_i)_n$ by indirect detection works best on samples that are free of salts besides $(P_i)_n$. The following steps were useful for preparing samples free of additional anions, originating from either synthesis or preparative separation: (i) adsorption to anion-exchange resin; (ii) elution of anions other than $(P_i)_n$ (e.g., Cl^-) with 0.1 M Na_2CO_3; (iii) elution of $(P_i)_n$ with concentrated 2.0 M NH_4HCO_3; (iv) removal of NH_4HCO_3 under vacuum.[68]

Analytes: Authentic Standards of $(P_i)_n$ ($n = 1-10$). Commercially available oligophosphates of a single chain length are limited to P_1, P_2, P_3, and *cyclo*-P_3. Preparative-scale separation of a mixture of $(P_i)_n$ (117% polyphosphoric acid[69]), by anion-exchange chromatography, generated samples of purified $(P_i)_n$, $n = 4-10$, that served as analytical standards. By analyzing standards added to mixtures of $(P_i)_n$, we identified peaks representing $(P_i)_n$ with $n = 1-10$.

Analytes: Mixtures of $(P_i)_n$. We demonstrated the resolution of the method by analyzing commercially available samples of higher $(P_i)_n$, covering a range of average length ($\bar{n}$): 117% polyphosphoric acid, $\bar{n} = 17$, $\bar{n} = 21$, $\bar{n} = 48$, and $\bar{n} = 65$. In addition, we prepared samples of $(P_i)_n$ generated by the dehydration of $NH_4H_2PO_4$ in mixtures with urea, using conditions reported by Orgel et.al., which were presumed to be plausible in prebiotic chemistry.[8,21]

Internal standard(s). We added an internal standard, $K^+CH_3SO_3^-$, 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 $(P_i)_n$ relative to that of a standard ($CH_3SO_3^-$) ($\mu_{P,rel}$ in eq 1); (iii) determine the relative and absolute concentrations of resolved $(P_i)_n$, by comparing the areas of peaks for $CH_3SO_3^-$ and $(P_i)_n$. In eq 1, $t_{CH_3SO_3^-}$ and t_P are the retention times for $CH_3SO_3^-$ and $(P_i)_n$, V is the voltage, L_1 is the length of the capillary, and L_2 is the distance between the inlet and detector.

$$\mu_{P,rel} = \mu_P - \mu_{CH_3SO_3^-} = \frac{L_1 L_2}{V}\left(\frac{1}{t_{CH_3SO_3^-}} - \frac{1}{t_P}\right) \qquad (1)$$

The electrophoretic mobility of $CH_3SO_3^-$ in free solution was near that of $(P_i)_n$, but peaks for $CH_3SO_3^-$ and $(P_i)_n$ did not overlap ($\mu_{CH_3SO_3^-} = 26.7$ cm^2 kV^{-1} min^{-1}). For experiments requiring additional internal standards, we also used Cl^-, $CF_3CO_2^-$, and $CH_3-C_6H_4-SO_3^-$.

RESULTS AND DISCUSSION

Resolution and Detection of Lower Oligophosphates by CZE. We analyzed mixtures of P_1, P_2, P_3, and *cyclo*-P_3 by CZE, using coated capillaries and running buffer composed of 3.0 mM TP^{2-} and 18.0 mM bis−tris (pH = 6.9) or 24.0 mM tris (pH = 8.4);[70] data from these experiments are shown in the Supporting Information. The results demonstrated (i) the indirect detection of anions, enabled by the steady-state absorbance signal of TP^{2-} at 254 nm and (ii) mobilities in the order *cyclo*-P_3 > P_3 > P_2 > P_1 (at pH = 6.9) that correspond to 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).

Resolution of Mixtures of $(P_i)_n$ by CGE. The resolution of mixtures of $(P_i)_n$ with $n > 5$ required the use of a sieving gel. The best results were obtained with capillaries filled with solutions of 9.1% PDMA (w/v; average molecular weight 58.9 kDa) in 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 ultrahigh purity N_2 applied to the inlet) for 10 min. 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).

The upper trace in Figure 1A shows the separation of $(P_i)_n$ in 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); P_3 (2.0 mM) and $CH_3SO_3^-$ (2.0 mM) were added to serve as internal standards. For comparison, the lower trace in Figure 1A shows the analysis of the same sample by CZE.

We identified peaks for P_1, P_2, and P_3 (marked with a dotted line in Figure 1B) by comparing the traces in Figure 1A to those collected for samples containing standards added to the mixture. In CGE experiments, mobilities of $(P_i)_n$ with $n > 3$ are lower than that of $CH_3SO_3^-$. In CZE, the mobilities of all $(P_i)_n$ are greater than that of $CH_3SO_3^-$. The contrast in the order of mobility

(64) 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.

(65) Huang, X. H.; Gordon, M. J.; Zare, R. N. *Anal. Chem.* **1988**, *60*, 375–377.

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(69) 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.

(70) Capillaries modified by the method of Cretich[42] were used over the course of several months in more than 100 runs, without observable change in the retention times for analytical standards.

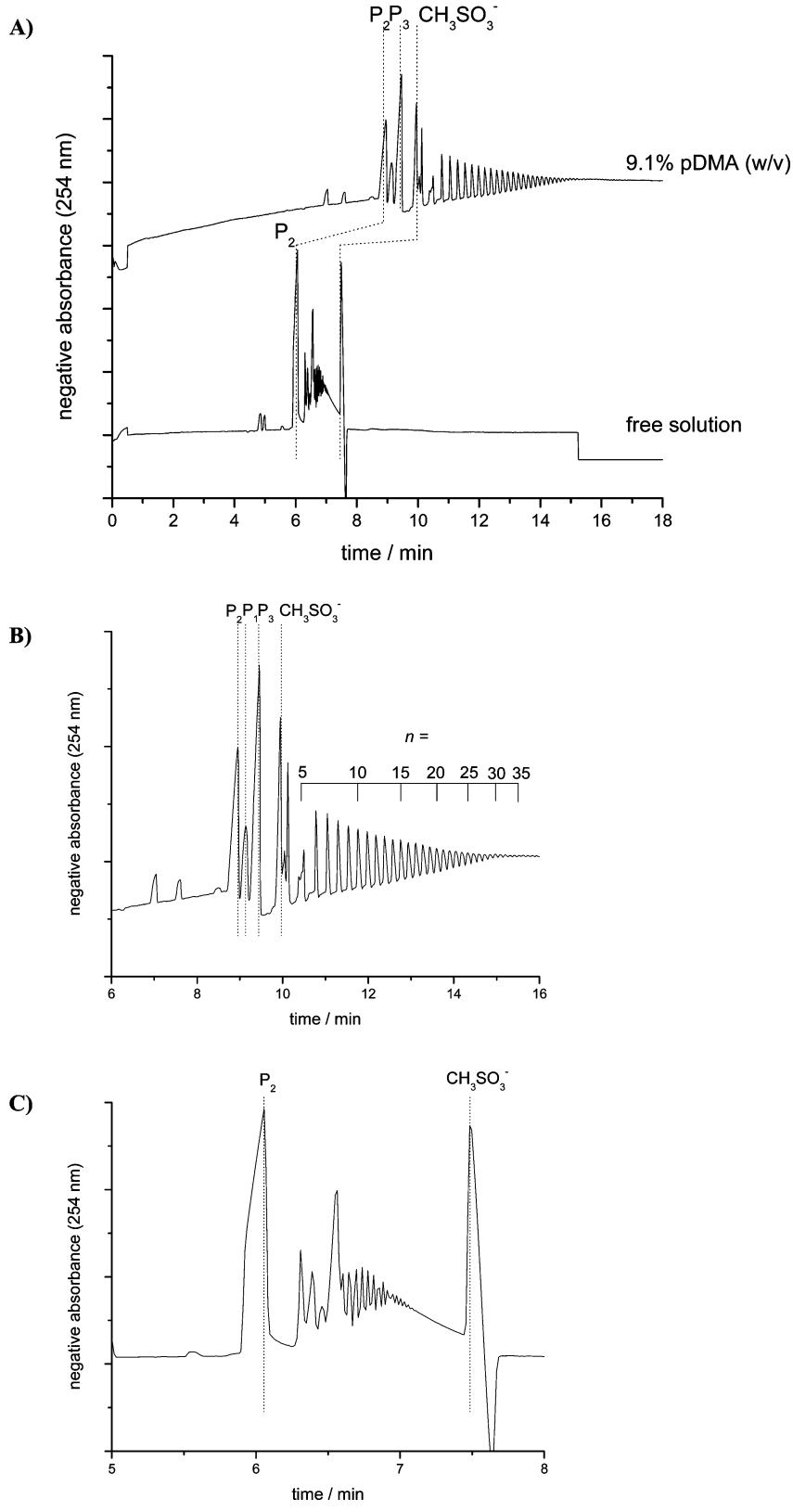

Figure 1. Resolution of $(P_i)_n$ by CGE. (A) Analysis of sodium polyphosphate (average chain length 17) in capillaries filled with 9.1% PDMA (w/v) gel (upper trace) or running buffer alone (24.0 mM tris, 3.0 mM terephthalic acid, pH = 8.4, 25 °C) (lower trace). Electrophoresis was performed by applying 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 A, respectively.

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 Figure 1B) suggests species as large as $\sim P_{35}$ in the sample.

Preparation of $(P_i)_n$, $n = 4-10$. Identification of peaks observed for broad distributions of $(P_i)_n$ required oligophosphate standards that are not commercially available. We separated a

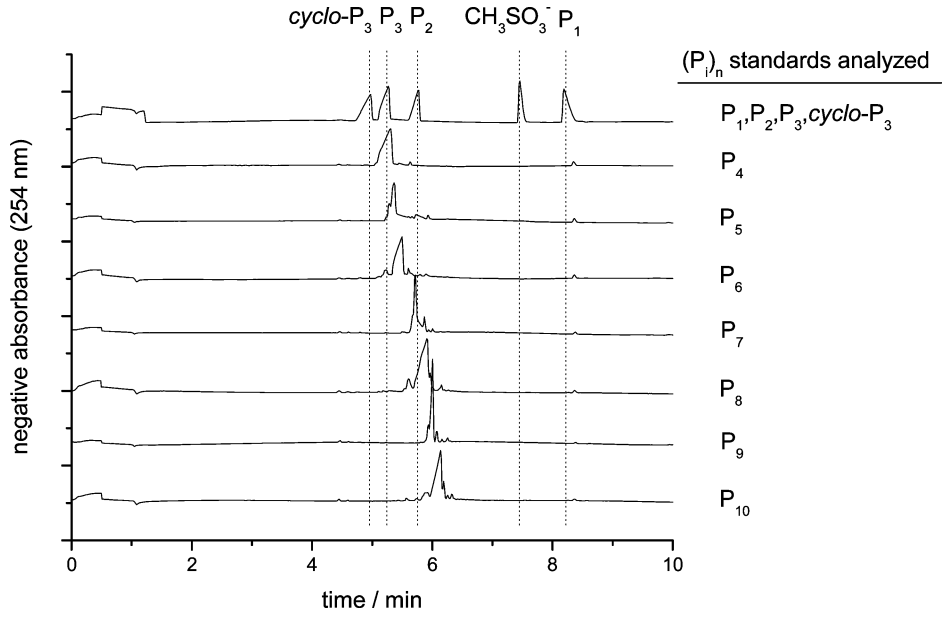

Figure 2. Standards of $(P_i)_n$, $n = 1-10$. CZE traces analyzing a mixture of commercially available P_1, P_2, P_3, *cyclo*-P_3, and $CH_3SO_3^-$ (top trace) and analytical standards P_4-P_{10} purified by anion-exchange chromatography (no internal standard added). CZE was performed by applying 14.6 kV across coated capillaries (57 cm in length, 50 cm from the inlet to detector) filled with running buffer composed of 18.0 mM bis−tris and 3.0 mM terephthalic acid (pH = 6.9, 25 °C).

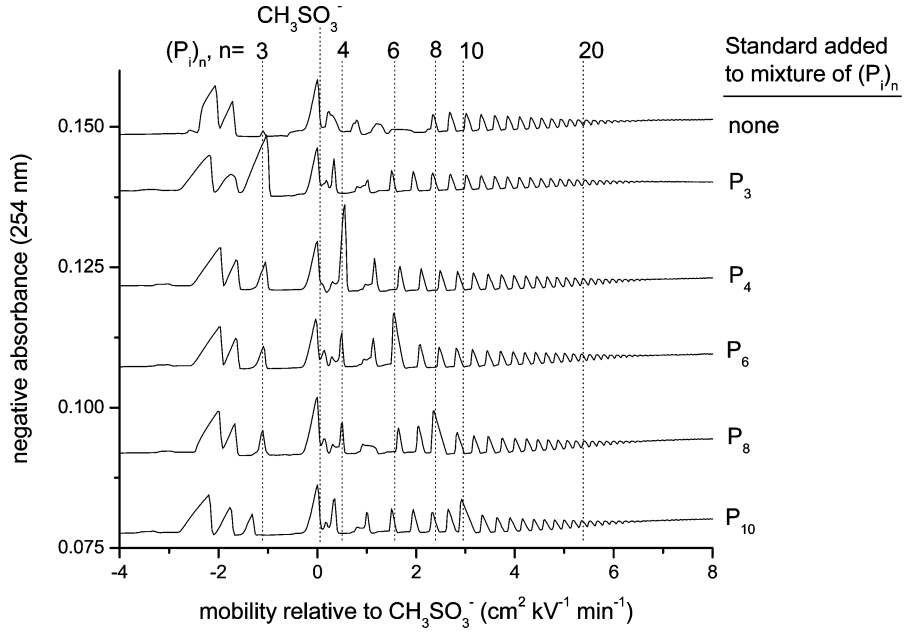

Figure 3. Identification of P_3-P_{10} in mixtures of $(P_i)_n$ by CGE. Traces are for the analysis of mixtures of $(P_i)_n$ (19 mM in total phosphate, pH ~ 8) with the addition of analytical standards for $(P_i)_n$ (2 mM). Mixtures were resolved by CGE at 14.6 kV using capillaries filled with 9.1% PDMA (w/v) and running buffer (24.0 mM tris, 3.0 mM TP^{2-}, pH = 8.4). Dotted lines mark peaks for species identified by added standards. Traces for samples with added P_7 or P_9 are available in the Supporting Information.

mixture of $(P_i)_n$ ($n \sim 1-10$, derived from 117% polyphosphoric acid[69]) 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_4HCO_3). Removal of NH_4HCO_3 by vacuum generated oligophosphate standards as the ammonium salt.

CZE traces analyzing purified $(P_i)_n$, $n = 4-10$, show one major peak in each sample (Figure 2). Small peaks next to the major peak and a small peak for P_1 (top trace in Figure 2 for reference) suggest small amounts of $(P_i)_{(n+1)}$ or $(P_i)_{(n-1)}$, and P_1. The relatively clean traces suggest that the conditions used in the preparation of samples do not cause extensive hydrolysis of $(P_i)_n$ or equilibration in chain length. Characterization of oligophosphate standards by ^{31}P NMR is available in the Supporting Information.

Identification of Peaks for $n = 3-10$ in Mixtures of $(P_i)_n$. Figure 3 shows CGE data analyzing mixtures of $(P_i)_n$ (19.0 mM

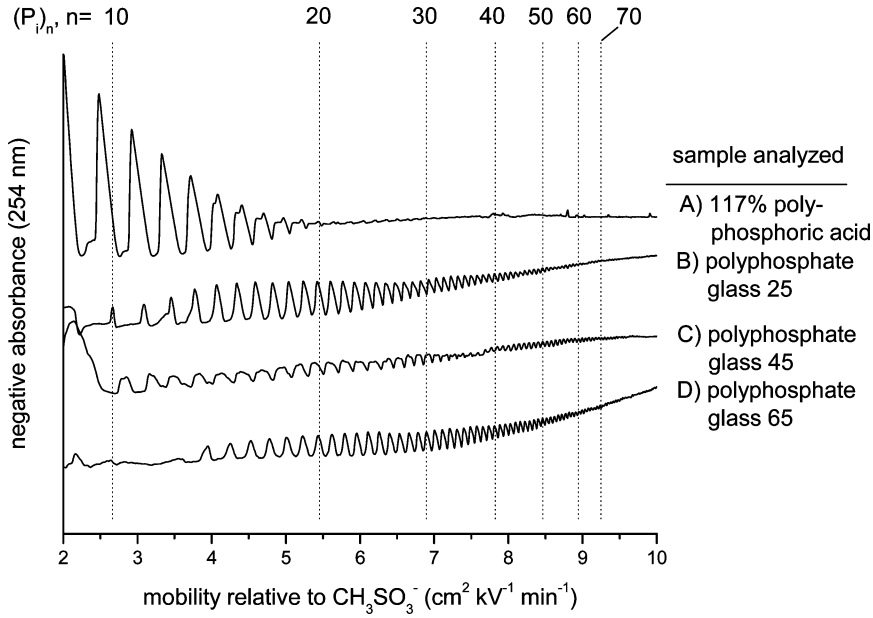

Figure 4. Resolution of commercially available mixtures of $(P_i)_n$. Samples consisting of commercially available mixtures of polyphosphate glass (20 mM in total phosphate, Na^+ salt) and $CH_3SO_3^-$ (2 mM) were analyzed by CGE. After electrokinetic injection (4.0 s at 10 kV), $(P_i)_n$ were separated 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). 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).[72]

in phosphate residues; $\bar{n} \sim 17$) and added P_3, P_4, P_6, P_8, or P_{10} (2.0 mM). The *x*-axis of traces in Figure 3 are in units of mobility relative to $CH_3SO_3^-$ $\mu_{p,rel}$ (eq 1). The traces show the alignment of peaks from run to run and allowed us to assign peaks with increased area to specific $(P_i)_n$ (dotted lines in Figure 3). The dotted line for $n = 20$ in Figure 3B is based on the reasonable assumption that the peaks continue in order of n.[71]

Mixtures of $(P_i)_n$ with n up to ~70. The CGE data in Figure 4 characterizes commercially available mixtures of $(P_i)_n$ with different distributions in chain length. Samples analyzed in traces A–D are in order of increasing average chain length. The results show that peaks with n up to ~70 can be resolved in a single run; longer $(P_i)_n$ may potentially be resolved using lower concentrations of PDMA.

Shapes of Peaks. The asymmetric peaks observed in both CZE and CGE experiments are typical for electropherograms collected by indirect UV absorbance. $(P_i)_n$ with mobility greater than that of TP^{2-} shows up as peaks broadened to the left, while $(P_i)_n$ with mobility less than that of TP^{2-} show up as peaks broadened to the right. These shapes are the results of dispersion by electromigration, originating from (i) differences in mobility between analytes and TP^{2-} and (ii) nonuniform electric fields inside sample zones. Discussion of the origin of the shapes of peaks is available in the Supporting Information, as well as in ref 59.

Quantitative Analysis of $(P_i)_n$: Areas of Peaks. Three contributions determine the area of a peak: (i) the response of $[TP^{2-}]$ 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 the areas of peaks for $(P_i)_n$ and $CH_3SO_3^-$ enables the quantification of resolved $(P_i)_n$. To account for the effect of (iii), areas of peaks are adjusted by the factor $(1/t_i)$, where t is the retention time of analyte i.[73] In eq 2, the ratio of adjusted areas for analyte i and $CH_3SO_3^-$ (A_i and $A_{CH_3SO_3^-}$) is related to the concentrations $[i]_S$ and $[CH_3SO_3^-]$ of the sample: z_i and $z_{CH_3SO_3^-}$ are the electrostatic charge of i and $CH_3SO_3^-$; $\mu_{CH_3SO_3^-}$ and μ_i are the mobilities of i and $CH_3SO_3^-$; μ_{C^+} is the mobility of the cation in the sample zone (bis–tris–H^+ or tris–H^+).

$$\frac{A_i}{A_{CH_3SO_3^-}} = \frac{[i]_S}{[CH_3SO_3^-]} \cdot \left[\frac{z_i}{z_{CH_3SO_3^-}}\right] \cdot \left[\frac{\mu_{C^+} + \mu_i}{\mu_{C^+} + \mu_{CH_3SO_3^-}}\right] \quad (2)$$

The Supporting Information contains our derivation of eq 2 and a discussion of the quantitative aspects of electrokinetic injection and indirect UV absorbance.

Equation 2 reveals the advantage of analyzing electropherograms by comparing peaks for $(P_i)_n$ and $CH_3SO_3^-$. The ratio $(A_i/A_{CH_3SO_3^-})$ depends on the ratio $([i]_S/[CH_3SO_3^-])$ 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 $CH_3SO_3^-$ by CZE demonstrated the relationship between peak areas and concentrations. Figure 5 shows values of $(A_{cyclo-P_3}/A_{CH_3SO_3^-})$ determined from the analysis of samples containing *cyclo*-P_3 and $CH_3SO_3^-$. Samples had ratios

(71) 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.

(72) Another possibility is that electrokinetic injections may result in concentrations of $(P_i)_n$ at the inlet that can lead to precipitation.

(73) Hilser, V. J.; Freire, E. *Anal. Biochem.* **1995**, *224*, 465–485.

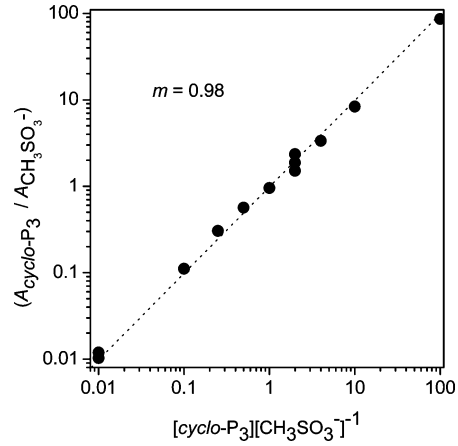

Figure 5. Quantitative calibration of *cyclo*-P_3. The plot shows values for $A_{cyclo-P_3}/A_{CH_3SO_3^-}$ determined in the analysis of *cyclo*-P_3 and $CH_3SO_3^-$ by CZE, using coated capillaries (57 cm in length, 50 cm between inlet and detector) filled with running buffer (18.0 mM bis−tris, 3.0 mM TP^{2-}, pH = 6.9). Points for ([*cyclo*-P_3]/[$CH_3SO_3^-$]) = 0.1, 1.0, or 10.0 are the average taken from eight experiments. Standard deviations for ($A_{cyclo-P_3}/A_{CH_3SO_3^-}$) are <5% of the measured values; error bars are not visible in plots with a logarithmic scale. Points at other values of [*cyclo*-P_3]/[$CH_3SO_3^-$] are from one or two trials. The slope *m* is for the line determined by fitting the data with adjusted weighting.[74]

of *cyclo*-P_3 to $CH_3SO_3^-$ that spanned a factor of 10^4. The results 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.[74] A value of 0.98 for the slope *m* is consistent with the complete ionization of all three acidic groups of *cyclo*-P_3 at pH = 6.8.

The calibration of ratios of peak areas to ratios of concentrations (of *cyclo*-P_3 and $CH_3SO_3^-$) provides a way to measure the amount of *cyclo*-P_3 in mixtures. Data collected for the quantitative analysis of P_1, P_2, and P_3 are available in the Supporting Information. Calibration of peak areas for $(P_i)_n$ of greater chain length should also be straightforward in CGE and enable the quantification of resolved $(P_i)_n$ in mixtures.

Analysis of $(P_i)_n$ Generated by Dehydrating P_i. The method developed in this paper should be useful for exploring reactions that may have generated $(P_i)_n$ on the prebiotic earth. CGE allowed us to characterize samples prepared by dehydrating P_i in mixtures with urea, using conditions originally reported by Orgel.[8,21,75] The mechanism of dehydration by urea has not been determined. Urea is, however, essential; heating $NH_4H_2PO_4$ in the absence of urea only generates a small amount of P_2.

We synthesized $(P_i)_n$ by evaporating solutions of $NH_4H_2PO_4$ (0.2 mmol) and urea (20.0 mmol) and heating to temperatures

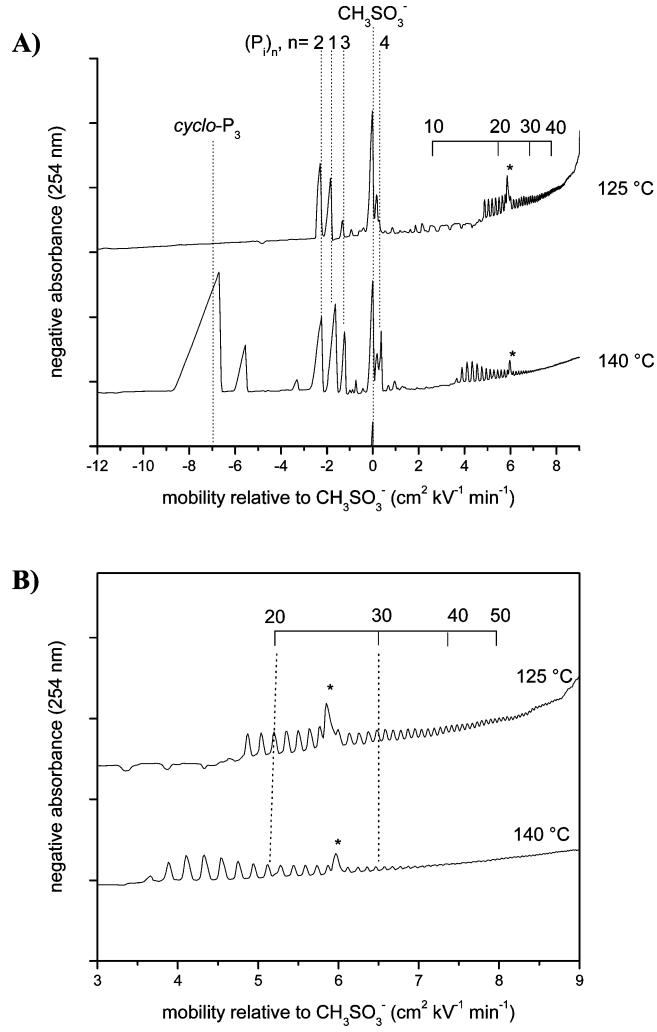

Figure 6. Analysis of $(P_i)_n$ generated under possible prebiotic conditions. (A) Samples of $(P_i)_n$, generated by heating mixtures of $NH_4H_2PO_4$ and urea, were 20.0 mM in total phosphate with pH ~ 7. Analysis by CGE used coated capillaries filled with solutions of PDMA (9.1% w/v) and running buffer (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 Figure 3. (B) Close-up views of peaks observed in the range μ = 3−9 $cm^2\ kV^{-1}\ min^{-1}$. 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 the specific sample (peaks for $n \sim 5-20$ appear sharp in the analysis of other mixtures using the same procedure (Figures 1 and 3)). One possible reason for peak broadening in some parts of the trace, but not others, is a nonuniform pH in the capillary during separation, resulting in electrodispersion. Irregularities in pH may originate from either (i) a mismatch in pH between running buffer and sample or (ii) bias toward the injection of the fully deprotonated form of $(P_i)_n$, resulting in a pH for the sample zone that is different from the pH of the sample.

≥125 °C. After 48 h of heating mixtures to either 125 or 140 °C, we cooled the samples to 25 °C, added water (2.0 mL), and removed insoluble material by centrifugation. Analysis of the samples by CGE (data in Figure 6) showed the formation of $(P_i)_n$ with $n > 40$ and distinguished the composition of mixtures generated at 125 and 140 °C. *Cyclo*-P_3 is the most abundant species in mixtures prepared at 140 °C. In contrast, the mixture prepared at 125 °C does not contain *cyclo*-P_3, despite containing linear $(P_i)_n$ with $n > 40$. The reason for the

(74) 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_{CH3SO3}) to log ($[i]_S/[CH_3SO_3^-]$) 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 2); y is (A_i/A_{CH3SO3}), x is ($[i]_S/[CH_3SO_3^-]$), and m is a proportionality constant obtained by fitting to the data.

(75) Handschuh, G.; Lohrmann, R.; Orgel, L. E. *J. Mol. Evol.* **1973**, *2*, 251–262.

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 polyphosphorylated compounds, likely depend on whether $(P_i)_n$ are cyclic or linear. 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 generate a triphosphate group similar to that of ATP. The resolution and convenience of CGE should enable a broad survey of conditions for the synthesis of $(P_i)_n$ and for reactions of $(P_i)_n$ with −OH groups. The results should be helpful in 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 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 P_1−P_{10} and resolution up to P_{70} validates a method that will be useful in studies of $(P_i)_n$ relevant to prebioitic chemistry and biochemistry.

In addition to characterizing the composition of samples of $(P_i)_n$, the method we have developed is potentially useful for analyzing other anionic, oligomeric species without a sensitive chromophore. Examples relevant to prebiotic chemistry are oligomers of phosphate condensed with organic compounds (e.g., structures with formula $(PO_3^--RO)_n$ or $(P_2O_6^{2-}-RO)_n$)). 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. This material is available free of charge via the Internet at http://pubs.acs.org.

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