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SEPARATION OF POLYPHOSPHATES BY ANION EXCHANGE THIN LAYER CHROMATOGRAPHY

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SUMMARY

A rapid and simple anion exchange thin layer chromatographic technique for the separation of polyphosphates is described. The method allows separation of several linear and cyclical phosphates by one-dimensional development of thin layer plates and also permits estimation of the approximate chain lengths of the components of polyphosphate glasses.

INTRODUCTION

There has been considerable interest in the formation, degradation and characterization of polyphosphates in biological[1] and nonbiological systems[2]. Methods for estimating polyphosphates in biological systems depend upon analysis of polyphosphate as orthophosphate after strong acid hydrolyis. After extraction of polyphosphates from cells, attempts are often made to free the extracts of organic phosphates by removing nucleic acids and nucleotides by adsorption to charcoal[3]. Alternatively, polyphosphate is precipitated as the salt of a heavy metal[4].

In biological as well as nonbiological systems attempts have been made to resolve mixtures of polyphosphates, especially by paper partition chromatography. These techniques require long development times and often two-dimensional elutions[5–8]. Paper chromatography has been used to follow the kinetics of polyphosphate hydrolysis[2] and has been useful in resolving mixtures of polyphosphates of widely divergent chain lengths[9]. Partial separation of the components of a polyphosphate mixture using molecular-sieve column chromatography has been reported[10]. Anion exchange column chromatography has been used with success in separating polyphosphates up to chain length of about 15 (ref. 11).

There are several reports of partition chromatography upon various types of thin layers. RÖSSEL[12] achieved good separations of linear polyphosphates of up to eight phosphorus atoms per molecule. Cyclical phosphates were most readily separated from linear phosphates by two-dimensional development. At nearly the same time

AURENGE, DEGEORGES AND NORMAND[13] reported good separations of linear polyphosphates of up to eight phosphorus atoms per molecule. Several of the oligomeric components of Graham's salt were separated. Cyclical polyphosphates were separated using developing solutions which were unable to separate linear polyphosphates. CLESCERI AND LEE[14] separated ortho- from pyrophosphate by partition chromatography on thin layers. Good separations of linear and cyclical polyphosphates were achieved using ascending and circular thin layer techniques[15]. Others have reported partition chromatography on thin layers to be variously successful[16,17]. Recently, BERGER, MEYNIEL AND PETIT[18] reported successful separation of ortho-, pyro- and tripolyphosphate on ion exchange Biorex 5 (Cl^-) thin layers.

This report describes a rapid and simple anion exchange thin layer chromatographic technique for the separation of polyphosphates. The method allows simultaneous chromatography of large numbers of samples and estimation of the approximate chain lengths of components of polyphosphate mixtures.

EXPERIMENTAL

Polyethyleneimine (PEI) impregnated micro-crystalline cellulose (Avicel) coated thin layer plates (250 μ thickness) were employed (Analtech Inc., Wilmington, Del.). Plates were stored at 4°. As suggested by RANDERATH[19], lines were scratched in the thin layers, about 1.0 mm wide, extending from just below the origin to the bottom edge of the plate. Then the plates were predeveloped in distilled water and dried at room temperature.

Aqueous solutions of 2.5 mg/ml of various sodium salts of phosphates were spotted such that 0.20–0.25 μg of the salt was applied to the thin layer. The plates were placed in covered battery jars (20 × 8.5 × 21 cm) containing 300 ml of the following concentrations of LiCl: 0.05 *M*, 0.10 *M*, 0.30 *M*, 0.40 *M*, 0.50 *M*, 1.0 *M*, 1.6 *M*, or 3.0 *M*. The solvent front was allowed to advance about 10 cm past the origin at room temperature (22–25°). This required about 30 min. The plates were removed from the jars and allowed to air dry.

The location of phosphorus-containing spots was revealed after spraying with the reagent of HANES AND ISHERWOOD[20]. Freshly sprayed plates were placed horizontally upon a heavy glass slab in an oven preheated to 150° until the thin layer dried. In this way polyphosphate hydrolysis was accelerated while the diffusion of spots was minimized. It was often necessary to repeat the spraying and drying procedure when plates had been developed with higher concentrations of LiCl. After reduction of phosphomolybdate to molybdenum blue under ultraviolet light[21], plates were exposed to NH_3 vapors. Blue spots on a white background were readily marked. The color does not appreciably fade with time.

The following sodium salts of phosphate compounds were used as standards. Dibasic sodium phosphate (Na_2HPO_4), and sodium pyrophosphate ($Na_4P_2O_7 \cdot 10\ H_2O$) were analytical reagent grade and standard items of commerce. So-called sodium "hexametaphosphate" glass was supplied by the Monsanto Company, St. Louis, Mo. Highly purified samples of sodium trimetaphosphate ($Na_3P_3O_9$), sodium tripolyphosphate ($Na_5P_3O_{10} \cdot 6\ H_2O$), sodium tetrametaphosphate ($Na_4P_4O_{12} \cdot 4\ H_2O$), sodium hexametaphosphate ($Na_6P_6O_{18}$), and four sodium phosphate glasses having mean chain lengths of 5, 10, 20 and 174, respectively, and having the general empirical

formula $Na_{n+2}P_nO_{3n+1}$, were supplied through the generosity of Dr. E. J. GRIFFITH, Inorganic Research Dept., Monsanto Company, St. Louis, Mo.

RESULTS AND DISCUSSION

Fig. 1 is a typical chromatogram of the various phosphate salts developed by 0.30 *M* LiCl. Table I gives the R_F values of the sodium salts of ortho-, pyro-, tripoly, trimeta-, tetrameta- and hexametaphosphates when PEI plates were developed by concentrations of LiCl ranging from 0.05–0.50 *M*. Best separations were obtained with concentrations of 0.10–0.30 *M* LiCl. At 0.50 *M* or greater LiCl concentrations, all of the highly purified polyphosphates tested demonstrated R_F values approximating that of orthophosphate. When PEI plates were developed in distilled water, none of the phosphates moved from the origin. When Avicel plates, not impregnated with PEI, were spotted and developed in distilled water, all of the phosphates migrated with the solvent front. Thus the chromatographic behavior of the various phosphate salts

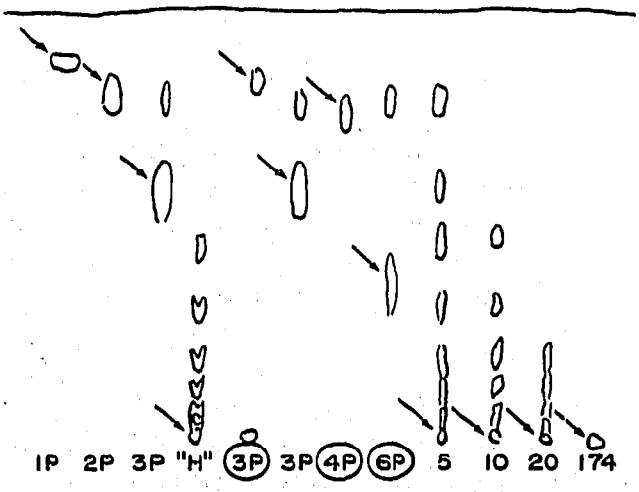


Fig. 1. Tracing of chromatogram of sodium salts of polyphosphates upon PEI impregnated Avicel thin layer plate developed with 0.3 *M* LiCl. Sodium salts of orthophosphate (1P), pyrophosphate (2P), tripolyphosphate (3P), so-called "hexametaphosphate" glass ("H"), trimetaphosphate ((3P)), tetrametaphosphate ((4P)), and hexametaphosphate ((6P)) as well as glasses having mean chain lengths of 5, 10, 20 and 174 were chromatographed. Arrows point to the developed spots which are of greatest color intensity.

TABLE I

R_F VALUES OF HIGHLY PURIFIED PHOSPHATE SALTS WHEN PEI IMPREGNATED AVICEL THIN LAYER PLATES WERE DEVELOPED IN VARIOUS MOLAR CONCENTRATIONS OF LiCl

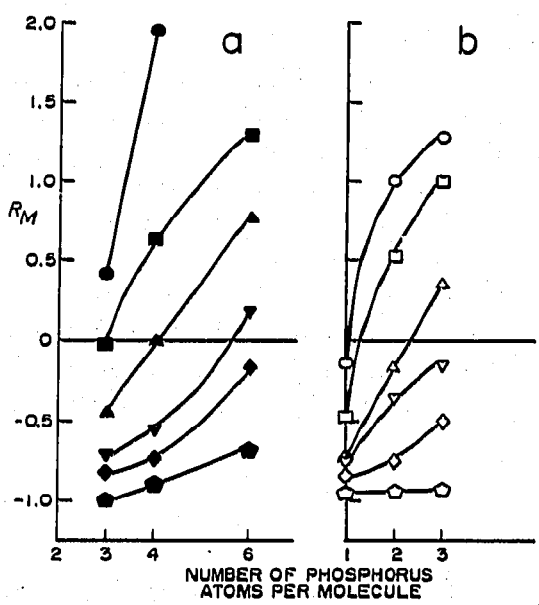
Phosphate salt	*Molar concentration of LiCl*					
	0.05	*0.10*	*0.20*	*0.30*	*0.40*	*0.50*
Orthophosphate	0.58	0.75	0.84	0.85	0.88	0.90
Pyrophosphate	0.09	0.24	0.60	0.79	0.85	0.89
Tripolyphosphate	0.05	0.09	0.30	0.60	0.76	0.87
Trimetaphosphate	0.28	0.52	0.74	0.84	0.87	0.91
Tetrametaphosphate	0.06	0.20	0.50	0.79	0.84	0.89
Hexametaphosphate	0.00	0.05	0.15	0.39	0.59	0.83

was in accord with their anionic character and the anion-exchange quality of the thin layer plate rather than being a reflection of the relative solubility of the salts in aqueous solution.

Chromatograms developed with 0.05–0.40 *M* LiCl revealed that tripolyphosphate and hexametaphosphate samples contained an appreciable contaminant having an R_F value consistent with its identification as pyrophosphate. The hexametaphosphate salt also contained a contaminant not mobilized by 0.10 *M* LiCl while the trimetaphosphate salt contained a contaminant which could not be mobilized by even the highest concentration of LiCl tested (3.0 *M*).

All phosphate residues of a cyclic polymer should be anionically equivalent. If the chromatographic behavior of cyclical polyphosphates is merely a function of the number of phosphate residues per molecule, then a plot of R_M, the log $(1/R_F - 1)$, *vs.* the number of P atoms per molecule for an homologous series ought to yield a straight line[22,23]. Such a plot (Fig. 2) shows that for the cyclical (a) and linear (b) phosphates no families of unequivocally straight lines are developed when various phosphates are chromatographed at different LiCl concentrations. Inability to accurately estimate very low R_F values may, however, produce large errors in R_M computation and obscure true linearity of some lines.

Fig. 3 gives the results of chromatographic analysis of four phosphate glasses whose mean chain length are 5, 10, 20 and 174, respectively, and of so-called "hexametaphosphate" glass, by five different concentrations of LiCl developing solution.



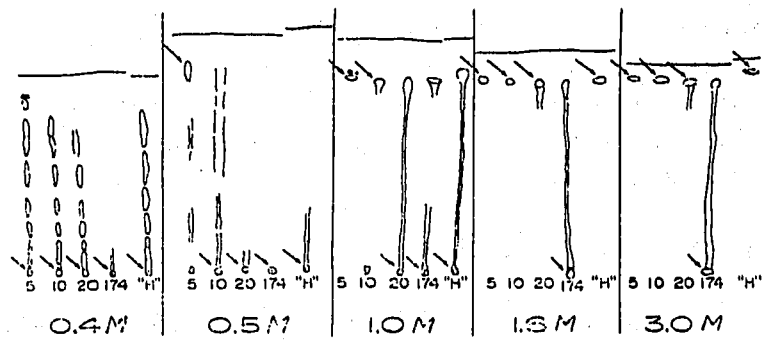


Fig. 2. Plot of R_M, the log $(1/R_F - 1)$, as a function of the number of phosphorus atoms per molecule at different concentrations of LiCl developing solution. In graph a the chromatographic behavior of trimeta-, tetrameta- and hexametaphosphate salts is presented using 0.05 *M* (●), 0.10 *M* (■), 0.20 *M* (▲), 0.30 *M* (▼), 0.40 *M* (◆) and 0.50 *M* (⬟) LiCl developing solution. In graph b the chromatographic behavior of ortho-, pyro- and tripolyphosphate salts is presented using 0.05 *M* (○), 0.10 *M* (□), 0.20 *M* (△), 0.30 *M* (▽), 0.40 *M* (◇) and 0.50 *M* (⬠) LiCl developing solution.

Fig. 3. Tracing of montage of chromatograms of polyphosphate glasses having mean chain lengths 5, 10, 20 and 174 and of so-called "hexametaphosphate" glass ("H"). The behavior in the following LiCl concentrations is shown: 0.4 *M*, 0.5 *M*, 1.0 *M*, 1.6 *M* and 3.0 *M*. Arrows point to the spots which have greatest color intensity.

Each of these phosphate glasses is a mixture of polyphosphates. Sometimes only a streak of molybdenum blue color could be discerned on the chromatogram; however, if greater amounts of phosphate glass were spotted, many intense blue spots could be delineated within the streak. Obviously, there are large numbers of components of these glasses.

It is possible to separate populations of polyphosphates of similar chain length. Fig. 3 shows that the major portion of a polyphosphate glass of mean chain length of 5 residues is not mobilized from the origin unless the chromatogram is developed with at least 0.50 *M* LiCl. Similarly, the major portion of a polyphosphate glass with a mean residue number of 10 is not mobilized unless 1.0 *M* LiCl is used. Mobilization of the major portion of a polyphosphate glass with a mean chain length of 20, and of the "hexametaphosphate" glass, requires development with at least 1.6 *M* LiCl, while the major portion of a polyphosphate glass with a mean chain length of 174 is not mobilized from the origin, even with 3.0 *M* LiCl. Hence, the relative chain length of an unknown polyphosphate can be estimated by development with concentrations of LiCl sufficient to mobilize a polyphosphate of known mean chain length but insufficient to mobilize a polyphosphate of greater mean chain length.

The behavior of polyphosphates upon these chromatograms appears to be a function of the negative charge which they bear, the anion exchange quality of PEI and the concentration (activity) of Cl^- in the developing solution. One cannot, however, completely exclude the influence of other factors upon chromatographic behavior.

We have found this chromatographic technique to be useful in characterizing the chain length of polyphosphates made by a microorganism. The method allows ready separation of inorganic polyphosphates from phosphate-containing organic compounds. It avoids the problems of non-differential adsorption of nucleotides and nucleic acids to charcoal and of incomplete precipitation of polyphosphates by heavy metals. When cells are prelabeled with ^{14}C, and then incubated with ^{32}P-orthophosphate, extracted in dilute alkali[24], and the extracts neutralized and chromatographed, one readily observes differential rates of migration of ^{14}C and ^{32}P radioactivity with various concentrations of LiCl developing solution. This behavior establishes the existence and relative chain length of biosynthetic polyphosphates. The amounts of newly formed polyphosphate are readily quantitated by evaluation of ^{32}P radioactivity.

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