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CHROMATOGRAPHIC SEPARATION OF CYCLIC PHOSPHATES BY MEANS OF AN ANION-EXCHANGE DEXTRAN GEL

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SUMMARY

Chromatographic separation of cyclic phosphates on a Dowex 1 X4 resin or a QAE-Sephadex A-25 gel column was investigated. The distribution ratios of a series of cyclic phosphates from trimeta- to octametaphosphate were measured at various concentrations of potassium chloride, which was used as eluting agent. When the elution was carried out on a QAE-Sephadex A-25 column with 0.30 *M* potassium chloride, trimeta-, tetrameta-, pentameta- and heptametaphosphate were separated from each other and from hexameta- and octametaphosphate, but the latter two cyclic phosphates were eluted together. These two cyclic phosphates were separated by elution with 0.25 *M* potassium chloride.

INTRODUCTION

Graham's salt contains a series of cyclic phosphates including trimetaphosphate, which is the lowest member of the series, through octametaphosphate or higher members[1]. THILO AND SCHÜLKE[2] have demonstrated that the total content of these cyclic phosphates in Graham's salt is about 10% and that the content of the individual cyclic phosphates decreases nearly exponentially with the increase in their degrees of polymerization.

Of these cyclic phosphates, trimeta- and tetrametaphosphate are well known and can be easily prepared from various kinds of materials from different sources. GRIFFITH *et al.*[3] and SCHÜLKE[4] have succeeded in synthesizing sodium hexameta- and sodium octametaphosphate, respectively, in large amounts from starting materials other than Graham's salt. THILO AND SCHÜLKE[2] have prepared several grams of sodium pentameta- and sodium hexametaphosphate from Graham's salt.

The present investigation was undertaken to devise a method for the chromatographic separation of a series of cyclic phosphates from trimeta- to octametaphosphate by means of an anion-exchange dextran gel as well as to obtain fundamental information on the isolation of pentameta- and heptametaphosphate in large amounts from the mixture of cyclic phosphates contained in Graham's salt.

Various methods have been employed in order to separate individual cyclic

phosphates. These methods are divided into two groups: precipitation and chromatography. As for the precipitation, THILO AND SCHÜLKE[2] have used fractional precipitation with acetone and hexaminecobalt(III) chloride to concentrate cyclic phosphates in Graham's salt and then to isolate pentameta- and hexametaphosphate. However, it is difficult to separate the individual cyclic phosphates from each other in high purity by this method.

Various chromatographic methods have been investigated for the analysis of condensed phosphates. A series of cyclic phosphates with degrees of polymerization of three to eight can be easily identified in the presence of linear phosphates[1] by two-dimensional paper chromatography. OOIJ *et al.*[5] have used thin-layer chromatography to separate cyclic phosphates and other oxo anions of phosphorus. IIDA *et al.*[6] have investigated the fundamental behavior of cyclic and linear phosphates in thin-layer chromatography. Paper electrophoresis has been successfully employed for the separation of trimeta- and tetrametaphosphate from each other and from linear phosphates[7]. Although the gel chromatographic behavior of trimeta- and tetrametaphosphate and other oxo acids of phosphorus has been investigated[8–10], it is difficult to separate these two cyclic phosphates by this method.

Ion-exchange chromatography is best for the separation of a series of similar ionic species. It has been demonstrated by several investigators[11–13] that trimeta- and tetrametaphosphate can be separated by ion-exchange chromatography. ROTHBART *et al.*[13] have indicated that trimeta-, tetrameta- and probably pentametaphosphate and a series of linear phosphates from ortho- to tridecaphosphate can be separated from each other on an Amberlite IRA-400 resin column. In a previous paper[14] the present authors have demonstrated that trimeta-, tetrameta- and pentametaphosphate can be completely separated from each other by the use of an Amberlite IRA-400 resin column, but hexameta- and heptametaphosphate are eluted together. The elution behavior of octametaphosphate on this column has remained unsolved. These investigations have shown that a series of cyclic phosphates from trimeta- to hexameta- or heptametaphosphate are eluted in the decreasing order of their molecular weights from the anion-exchange resin column under the conditions employed. On the other hand, the studies[10] on the gel chromatographic behavior of oxo acids of phosphorus suggest that cyclic phosphates may also be eluted in the same order from a Sephadex gel column under suitable conditions. Therefore, expecting a co-operating effect between ion-exchange chromatography and gel chromatography, the present authors attempted to use an anion-exchange gel for the separation of cyclic phosphates. QAE-Sephadex A-25, which is produced by combining tertiary alkyl ammonium groups with a dextran gel, was employed in this work.

It was found that the members of the series of cyclic phosphates from trimeta- to octametaphosphate can be separated from each other by means of two sets of chromatographic runs. From these results it would be expected that large quantities of pentameta- and heptametaphosphate will be isolated from a mixture of cyclic phosphates by this method. For sake of comparison, the separation of cyclic phosphates with an anion-exchange resin, Dowex 1 X4 was also examined.

Trimeta-, tetrameta-, pentameta-, hexameta-, heptameta- and octametaphosphates are represented by P_{3m}, P_{4m}, P_{5m}, P_{6m}, P_{7m} and P_{8m}, respectively, in the figures and tables of this paper.

EXPERIMENTAL

Preparation of trimeta-, tetrameta, hexameta- and octametaphosphate

Sodium trimetaphosphate hexahydrate, $Na_3P_3O_9 \cdot 6H_2O$ was produced by heating Graham's salt at 520° for 12 h, cooling slowly to room temperature and then crystallizing from an aqueous solution[15].

Sodium tetrametaphosphate tetrahydrate, $Na_4P_4O_{12} \cdot 4H_2O$ was prepared by treating its copper salt with a sod um sulfide solution[15].

Sodium hexametaphosphate hexahydrate $Na_6P_6O_{18} \cdot 6H_2O$ was synthesized by GRIFFITH's method[3]. A mixture of lithium carbonate and orthophosphoric acid with an Li_2O/P_2O_5 ratio of about 7:5 was heated at 200° for 1 h and then at 275° for 5 h. Sodium hexametaphosphate was isolated from the resulting product.

Sodium octametaphosphate hexahydrate, $Na_8P_8O_{24} \cdot 6H_2O$ was prepared by SCHÜLKE's method[4]. Lead tetrametaphosphate tetrahydrate, which was obtained from sodium tetrametaphosphate and lead nitrate, was heated at 150° for 2 h and then at 350° for 1 h. The product was converted to the sodium salt by treating it with a sodium sulfide solution and fractionating with ethanol.

Identification of the cyclic phosphates thus obtained was carried out by paper chromatography, X-ray diffractometry and acid–base titration.

Paper chromatography

The basic solvent used in this study was made by mixing 20 ml of 2-propanol, 20 ml of N,N-dimethylformamide, 20 ml of methyl ethyl ketone, 39.1 ml of water and 0.9 ml of concentrated aqueous ammonia. The filter paper used was Toyo Roshi Sheets No. 51A, one side of which was cut into tongue-shaped ends. Development was performed at 6° for about 10 h by the ascending method.

Measurement of distribution ratios

Distribution ratios for the Dowex 1 X4 resins were measured by the usual method at room temperature. About 0.5 g of the air-dried resin in the Cl^- form was put in a stoppered Erlenmeyer flask, and then 25 ml of an eluent, *i.e.*, 0.20–0.50 *M* potassium chloride buffered with acetate to pH 5.2, and 2 ml of a solution containing a known amount of a cyclic phosphate (*ca.* 250 μg as P) was added. After equilibrium was reached, the phosphate in the solution or in the resin phase was determined colorimetrically with a molybdenum (V)–molybdenum (VI) reagent[16]. When the resin phase was analyzed, the mixture was filtered by suction and the phosphate adsorbed in the resin was eluted with 0.5 *M* hydrochloric acid and then determined.

It is desirable to analyze a resin phase for the determination of a distribution ratio in a high eluent-concentration region where the distribution ratio is low. However, it is difficult to separate the resin completely from the solution by usual methods. Therefore, the improved method described below was applied to the measurement of distribution ratios for the QAE-Sephadex A-25 gel.

About 0.5 g of the air-dried gel in the Cl^- form was put into a small tube equipped with a sintered-glass disc and immersed in a mixture of the eluent solution (0.25–0.50 *M* KCl, pH 5.2) and the phosphate solution. After equilibration, most of the solution in the tube was filtered off by suction and the small amount of solution adhering on the gel particles was estimated by WAKI's method[17] which is based on

measurements of the amount of potassium chloride on and within the gel particles equilibrated with potassium chloride solutions at various concentrations. The phosphate adsorbed in the gel was eluted with 0.5 *M* hydrochloric acid and determined colorimetrically. In this work a volume distribution ratio, D_v, was used. D_v is defined as the amount of phosphorus per cm^3 of an exchanger bed divided by that per cm^3 of the solution. 1 g of air-dried Dowex 1 X4 resin or QAE-Sephadex A-25 gel occupies about 2.9 cm^3 or 5.5 cm^3, respectively, when immersed in 0.30 *M* potassium chloride.

For the measurement of distribution ratios of pentameta- and heptametaphosphate, the samples separated on a QAE-Sephadex A-25 column were used.

Elution procedure

The Dowex 1 X4 resin was washed with a strong base and strong acid repeatedly and finally converted to the chloride form. The QAE-Sephadex A-25 gel was successively washed with 0.5 *M* sodium hydroxide, 0.5 *M* hydrochloric acid and deionized water. The resin or the gel was then equilibrated with a given eluent. A column, 66 cm in length and 1.3 cm in diameter, was used for the elution with the Dowex 1 X4 resin (100–200 mesh) and a column, 88 cm in length and 1.5 cm in diameter for that with the QAE-Sephadex A-25 gel. 0.30 *M* or 0.25 *M* potassium chloride buffered to pH 5.2 with acetate was used as eluent. Each fraction of the effluent was collected with an automatic fraction collector and analyzed for phosphorus. All chromatographic runs were carried out at room temperature.

Instruments

X-ray diffraction patterns for powdered samples of hexameta- and octametaphosphate were obtained with a Rigaku Denki X-ray diffractometer Model D-3F and a Rigaku Denki multiposition-focusing camera. In order to examine the formation of octametaphosphate, thermal analysis of lead tetrametaphosphate tetrahydrate was carried out at a heating rate of 5°/min in static air with a Rigaku Denki differential thermal balance. Acid–base titration was done with a Hirama automatic recording titrator.

RESULTS AND DISCUSSION

Identification of the hexameta- and the octametaphosphate prepared in this work was carried out by paper chromatography, X-ray diffractometry and acid–base titration. Both the hexameta- and the octametaphosphate showed reasonable R_F values in comparison with the paper chromatogram for the mixture of the series of cyclic phosphates from trimeta- to octametaphosphate or higher members, which was fractionated from Graham's salt. The X-ray diffraction patterns for the powdered samples of both the cyclic phosphates, $Na_6P_6O_{18}\cdot 6H_2O$ and $Na_8P_8O_{24}\cdot 6H_2O$ were in good agreement with those obtained by GRIFFITH *et al.*[3] and SCHÜLKE[4], respectively. The acid–base titration curves for the hexameta- and the octametaphosphate showed only one jump, indicating cyclic structure.

Pentameta- and heptametaphosphate were isolated from the mixture of cyclic phosphates contained in Graham's salt by the method of chromatography devised in this study. Identification of these cyclic phosphates was carried out by paper chroma-

tography. The fractions of the effluent which were expected to contain pentameta- and heptametaphosphate were collected and concentrated *in vacuo*. Since the resulting solutions were nearly saturated with potassium chloride, the greater part of the potassium chloride was eliminated by dialysis. After this procedure, the pentameta- and heptametaphosphate were paper-chromatographed with the basic solvent. The resulting paper chromatograms are shown in Fig. 1. Peak C in the elution curve of Fig. 5 gave a spot in the region between the spots of tetrameta- and hexametaphosphate. Similarly, peak A gave a spot in the region between the spots of hexameta-

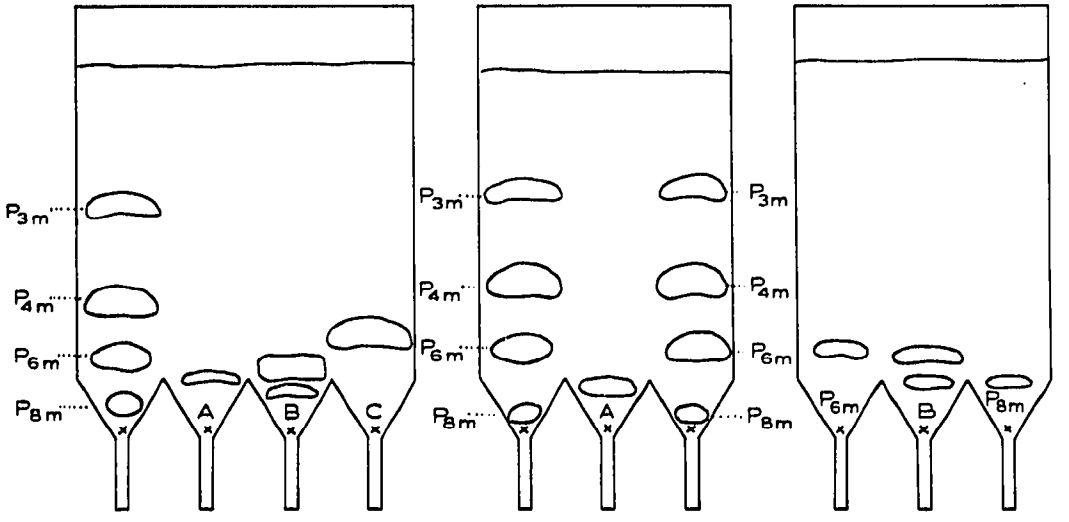

Fig. 1. Paper chromatograms. Samples A, B and C correspond to elution peaks A, B and C, respectively, in Fig. 5(C).

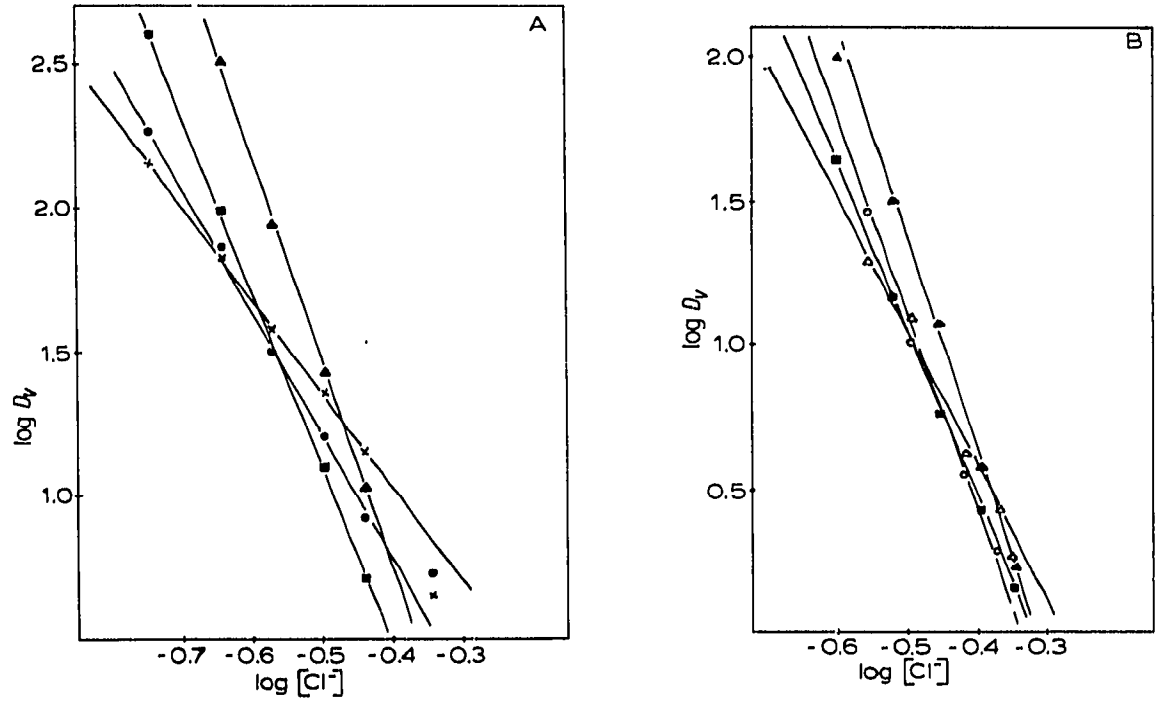

Fig. 2(A) and (B). Log D_v *versus* log [Cl⁻] for a series of cyclic phosphates using the Dowex 1 X4 resin. ×—×, P_{3m}; ●—●, P_{4m}; △—△, P_{5m}; ■—■, P_{6m}; ○—○, P_{7m}; ▲—▲, P_{8m}.

and octametaphosphate. These facts indicate that peaks C and A correspond to pentameta- and heptametaphosphate, respectively. As will be mentioned later, the charges of trimeta-, tetrametaphosphate, the peak C component, hexametaphosphate, the peak A component and octametaphosphate in the resin or the gel phase increased uniformly in this order. This also supports the fact that the identification mentioned above is correct.

Distribution ratios of the cyclic phosphates were measured in order to determine the eluent concentration for the best separation. Plots of the logarithms of the distribution ratios *versus* those of the eluent concentrations for the Dowex 1 X4 resins are shown in Fig. 2. The data in Fig. 2(A) and those in Fig. 2(B) were obtained using the resins with different lot numbers. The slight differences between the D_v values of

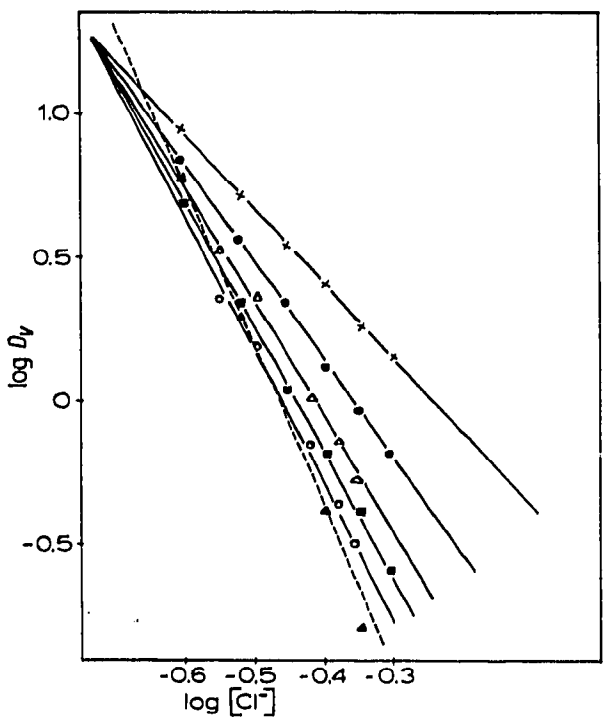

Fig. 3. Log D_v *versus* log [Cl⁻] for a series of cyclic phosphates using the QAE-Sephadex A-25 gel. ×—×, P_{3m}; ●—●, P_{4m}; △—△, P_{5m}; ■—■, P_{6m}; ○—○, P_{7m}; ▲---▲, P_{8m}.

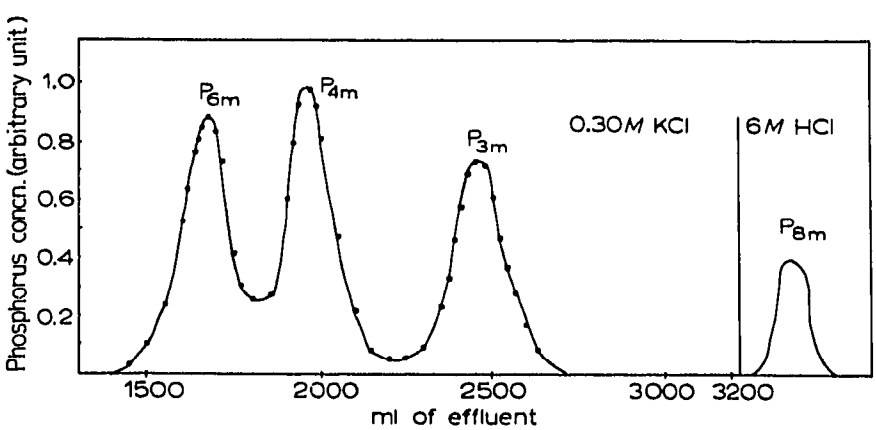

Fig. 4. Elution curve for a mixture of P_{3m}, P_{4m}, P_{6m} and P_{8m} obtained by the use of the Dowex 1 X4 resin column. Eluents: 0.30 *M* KCl and 6 *M* HCl.

hexameta- and octametaphosphate in Figs. 2(A) and 2(B) are probably due to the difference of the lot numbers. The distribution ratios at various eluent concentrations for the QAE-Sephadex A-25 gel are plotted in Fig. 3. Both of the plots for octametaphosphate in Figs. 2 and 3 show a somewhat irregular tendency in comparison with those for the other cyclic phosphates. The reason for this phenomenon has not been solved.

In general, the eluent concentration should be chosen so that a ratio of the distribution ratios of a given couple of the sample solutes attains a high value However, if the distribution ratios are too high, the elution will take a very long time. As a result of the above considerations, 0.30 *M* potassium chloride was chosen as an eluent. The logarithm of 0.30 is —0.52. The elution curve of a mixture of trimeta-, tetrameta-, hexameta- and octametaphosphate with this eluent on the Dowex 1 X4 resin column is shown in Fig. 4. Octametaphosphate was eluted with 6 *M* hydrochloric acid, because its D_v value was high in 0.30 *M* potassium chloride. The separation of trimeta- and tetrametaphosphate is good, but the elution curves of tetrameta- and

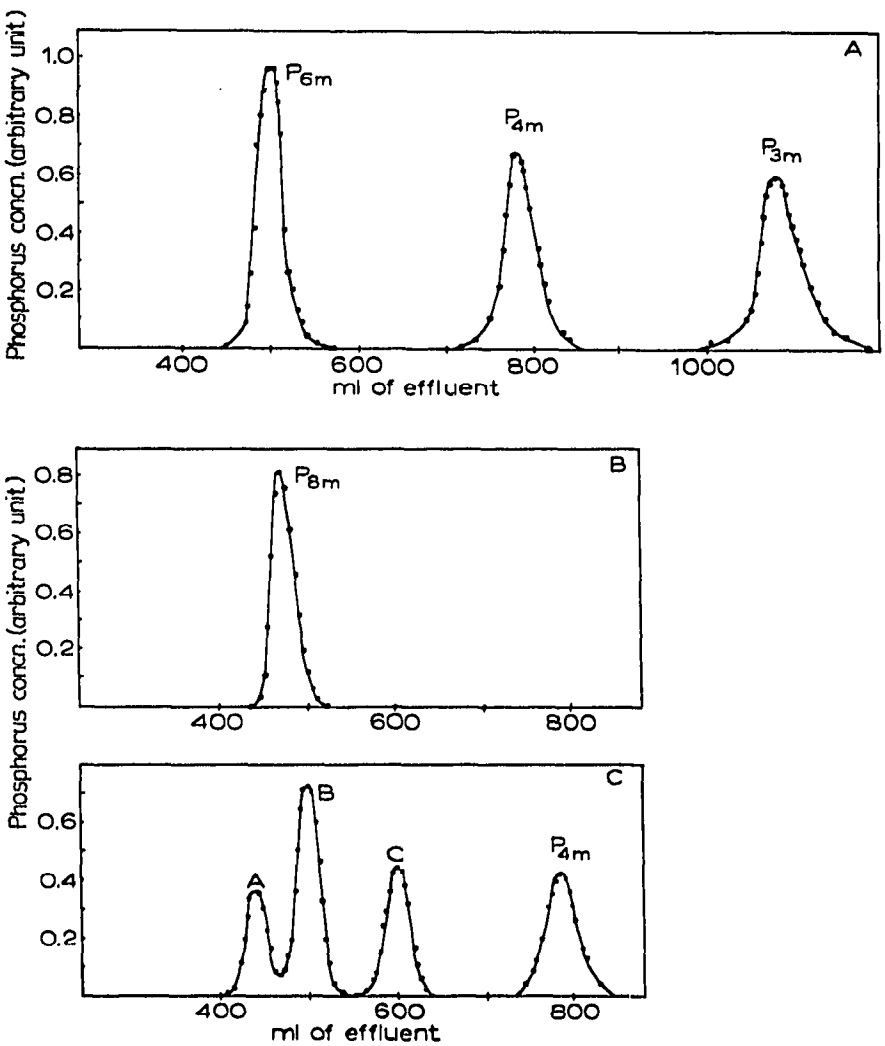

Fig. 5. Elution curves for cyclic phosphates obtained by the use of the QAE-Sephadex A-25 gel column. (A) P_{3m}, P_{4m}, and P_{6m}; (B) P_{8m}; (C) a mixture of cyclic phosphates fractionated from Graham's salt. Eluent: 0.30 *M* KCl.

hexametaphosphate overlap each other. It seems difficult to separate a series of cyclic phosphates from trimeta- to octametaphosphate under these conditions.

The elution curves of a mixture of trimeta-, tetrameta- and hexametaphosphate, octametaphosphate alone and a mixture of cyclic phosphates derived from Graham's salt with 0.30 *M* potassium chloride on the QAE-Sephadex A-25 column are shown in Fig. 5. As has been mentioned before, peaks C and A in Fig. 5 correspond to pentameta- and heptametaphosphate. The results indicate that trimeta-, tetrameta-, pentameta- and heptametaphosphate can be separated from each other and from hexameta- and octametaphosphate, but the latter two cyclic phosphates are eluted together under these conditions. However, the separation of these two species can be made with 0.25 *M* potassium chloride as shown in Fig. 6.

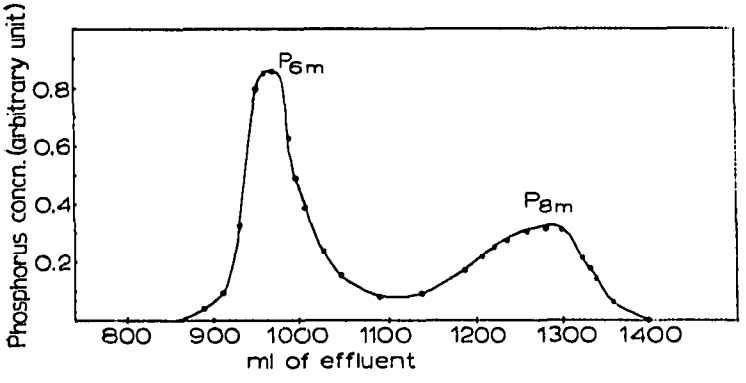

Fig. 6. Elution curve for a mixture of P_{6m} and P_{8m} obtained by the use of the QAE-Sephadex A-25 column. Eluent: 0.25 *M* KCl.

Resolution factors, R_s, were calculated in order to determine the efficiency of the separation. The resolution factor of components 1 and 2, $R_{s_2}{}^1$, is defined as $R_{s_2}{}^1 = (V_{e_1} - V_{e_2})/(2\sigma_1 + 2\sigma_2)$, where V_e is the elution peak volume of the component and σ is the peak width of the elution curve. The resolution factors thus obtained are shown in Table I. The column of the QAE-Sephadex A-25 gel was somewhat longer than that of the Dowex 1 X4 resin. Even if one takes this difference of the column length into consideration the results show that higher resolution is obtained with the QAE-Sephadex A-25 gel than with the Dowex 1 X4 resin. The broadening of the peak width for a given solute is determined by the particle size and the structure of the exchanger. Since the particle sizes of both the exchangers employed here were comparable, the difference in the resolution factors described above may be due to the difference in their structures rather than the difference in their particle sizes.

TABLE I

RESOLUTION FACTORS BETWEEN VARIOUS CYCLIC PHOSPHATES USING DOWEX 1 X4 RESIN OR QAE-SEPHADEX A-25 GEL

Exchangers	*Resolution factors, R_s*					
	P_{3m}-P_{4m}	P_{4m}-P_{5m}	P_{4m}-P_{6m}	P_{5m}-P_{6m}	P_{6m}-P_{7m}	P_{7m}-P_{8m}
Dowex 1 X4	1.8	—	1.0	—	—	—
QAE-Sephadex A-25	3.8	3.0	4.4	1.8	1.1	0.63

In other words the rate of the diffusion of a cyclic phosphate in the QAE-Sephadex A-25 gel may be faster than that in the Dowex 1 X4 resin.

As indicated in the earlier section of this paper, the molecular sieving effect seems to make some contribution to the separation of cyclic phosphates when the QAE-Sephadex A-25 column is used because the series of cyclic phosphates other than octametaphosphate are eluted in the decreasing order of their molecular weights and also a better separation is obtained than when the Dowex 1 X4 resin is used.

TABLE II

CHARGES OF CYCLIC PHOSPHATE ANIONS IN THE GEL PHASE, THE RESIN PHASE OR IN DILUTE AQUEOUS SOLUTION

Cyclic phosphates	*Dowex 1 X4*	*QAE-Sephadex A-25*	*In dilute aqueous solution*
P_{3m}	3.3	3.0	3.0
P_{4m}	4.4	4.0	4.0
P_{5m}	5.0	4.5	5.0
P_{6m}	6.0	5.0	6.0
P_{7m}	7.0	5.5	7.0
P_{8m}	7.6	6.5	8.0

As shown in Figs. 2 and 3, the plots of log D_v against log [Cl^-] form a straight line, the slope of which gives the charge of each cyclic phosphate in the exchanger phase. The charges thus obtained are tabulated in Table II. The values in the fourth column of Table II represent the charge of each cyclic phosphate in a solution where no ion-pair formation is assumed. The charges of the cyclic phosphates in the Dowex 1 X4 resin phase are in good agreement with those in the fourth column. However, pentametaphosphate and the higher members in the QAE-Sephadex A-25 gel phase carry considerably lower charges than those in the solution. In order to clarify the reason for this phenomenon, a further investigation is required.

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