CHROMATOGRAPHY OF PHOSPHORUS OXOACIDS

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ABSTRACT

The present state of studies on the chromatographic separation of phosphorus oxoacids is surveyed. In this paper, chromatographic techniques are divided into four groups, i.e. paper and thin-layer chromatography, paper electrophoresis, ion-exchange chromatography, and gel chromatography. The separation mechanisms and characteristics for these chromatographic methods are discussed and some examples for the separation of phosphorus oxoacids are described. As examples of the application of ion-exchange and gel chromatography, studies on the hot atom chemistry of ^{32}P in solid inorganic phosphates and those on the substitution reactions between diphosphonate (diphosphite) and polyphosphates are reported.

INTRODUCTION

Separation is one of the most important problems in any fields of pure and applied chemistry. Especially in basic research, when one wants to develop the chemistry of a certain group of new compounds, one encounters the problem how to separate these compounds. In other words, when a proper method of separation is established for a given group of new compounds, the chemistry for these substances will be rapidly developed.

There are many kinds of separation methods, for instance, precipitation, distillation, solvent extraction, ion exchange, etc. When a sample is composed of a small number of the compounds which have rather different chemical properties, these compounds can be easily separated by one of the separation methods mentioned above. However, when a sample is composed of the compounds which are similar in their chemical properties, the separation of them in general cannot be performed by such a one-step separation method. Especially when a number of similar compounds are contained in a sample, it is quite impossible to separate them by any one-step separation methods. In such a case we usually adopt one of the chromatographic techniques, which involve multi-step separation processes.

In the recent development of chemistry it is evident that chromatography has played many important roles. In the field of phosphorus compounds we can find a similar trend for the separation of these compounds.

Table 1 shows what kinds of chromatographic techniques are used for the separation of phosphorus compounds belonging to the respective groups,

Table 1. Chromatographic techniques used for the analysis of phosphorus compounds

	A	B	C	D	E	F	G	H
Elemental phosphorus	O							
Tervalent phosphorus compounds	O			O				
Phosphorus halides	O	O†		O†			O†	
Phosphorus oxoacids		O	O	O	O		O	O
Phosphorus–nitrogen compounds		O	O				O	
Cyclophosphazenes	O	O†		O		O		
Phosphorus–sulphur compounds	O	O					O	O†
Phosphoranes	O							
Sugar phosphates and sugar nucleotides		O	O	O	O		O	
Phosphorus lipids	O†	O		O		O	O	
Natural phosphorus–carbon compounds	O	O		O	O	O	O	O
Organophosphorus insecticides	O			O				

A, gas–liquid chromatography; B, paper chromatography; C, thin-layer chromatography (cellulose); D, thin-layer chromatography (SiO$_2$, Al$_2$O$_3$); E, paper electrophoresis; F, adsorption column chromatography (SiO$_2$, Al$_2$O$_3$); G, ion-exchange chromatography; H, gel chromatography.
† Hydrolysis or degradation products.

the classification of which is the same as that adopted by Halmann[1] in his book, *Analytical Chemistry of Phosphorus Compounds.* Gas–liquid chromatography is necessary for the analysis of cyclophosphazenes and organophosphorus insecticides. Paper chromatography plays important roles for the analysis of phosphorus oxoacids, phosphorus–nitrogen compounds, sugar phosphates, sugar nucleotides, and natural phosphorus–carbon compounds. Chromatography on a thin layer of silica is very often employed for the analysis of cyclophosphazenes, phosphorus lipids, natural phosphorus–carbon compounds, and organophosphorus insecticides. Ion-exchange chromatography is an indispensable method for the separation or preparation of each member in the groups of phosphorus oxoacids, sugar phosphates, and sugar nucleotides.

Now we will turn to the main subject of this paper, i.e. the chromatography of phosphorus oxoacids[2, 3]. The abbreviated notations for phosphorus oxoacids shown in *Table 2* will be used in the present paper.

Phosphorus oxoacids are classified into three groups as shown in *Table 3.* All phosphorus atoms in the oxoacids belonging to group A have an oxidation number of five. These oxoacids, except for orthophosphoric acid, are called condensed phosphoric acids and subdivided into three groups, namely linear, cyclic, and branched phosphates. Branched phosphates are in general stable only in a solid state and when they are dissolved in aqueous solution, they are decomposed into linear and/or cyclic phosphates. Therefore, branched phosphates are excluded from the scope of the present paper.

The phosphorus oxoacids belonging to group B contain one or more phosphorus atoms with an oxidation number lower than five, while those belonging to group C contain one or more phosphorus atoms with an oxidation number greater than five, i.e. phosphorus peroxoacids. The hitherto known phosphorus peroxoacids are peroxomono- and peroxodiphosphoric acids. Since we do not have any information on the chromatography of these peroxoacids, the following discussion is concerned only with groups A and B.

The separation methods for phosphorus oxoacids are classified into two

Table 2. Examples of abbreviated notations for phosphorus oxoacids or oxoanions

Abbreviated notations	Phosphorus oxoacids or oxoanions
P_1 or $\overset{5}{P}$	Monophosphate (ortho-)
P_2 or $\overset{5}{P}$—O—$\overset{5}{P}$	Diphosphate (pyro-)
P_3 or $\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$	Triphosphate
P_{3m} or $(-\overset{5}{P}-O-)_3$—ring	Trimetaphosphate
P_{4m} or $(-\overset{5}{P}-O-)_4$—ring	Tetrametaphosphate
$\overset{1}{P}$	Phosphinate (hypophosphite)
$\overset{3}{P}$	Phosphonate (phosphite)
$\overset{4}{P}-\overset{4}{P}$	Hypophosphate

For the lower entries, the abbreviated notations and corresponding structures:

$\overset{2}{P}-\overset{4}{P}$ corresponds to:

$$\begin{array}{ccc} & OH & OH \\ & | & | \\ H-P & — & P-OH \\ \| & & \| \\ O & & O \end{array}$$

$\overset{3}{P}-O-\overset{4}{P}-\overset{4}{P}$ corresponds to:

$$\begin{array}{ccccc} OH & & OH & & OH \\ | & & | & & | \\ H-P-O & -& P & -& P-OH \\ \| & & \| & & \| \\ O & & O & & O \end{array}$$

$(-\overset{3}{P}-)_6$—ring corresponds to:

$$\begin{array}{cccccc} OH & OH & OH & OH & OH & OH \\ | & | & | & | & | & | \\ P-&P-&P-&P-&P-&P \\ \| & \| & \| & \| & \| & \| \\ O & O & O & O & O & O \end{array}$$

categories, viz. precipitation and chromatography. The former is subdivided into two groups.

The first is precipitation of phosphorus oxoacids as their metal salts such as zinc, barium, and cobalt salts. For instance, when a zinc sulphate solution is added to a mixed solution of ortho-, pyro-, and triphosphate at pH 3.8, a precipitate of zinc pyrophosphate is obtained and ortho- and triphosphate remain in the solution. When a barium chloride solution is added to a mixed solution of ortho-, pyro-, tri, trimetaphosphate, and highly polymerized linear phosphates at pH 8.0, only trimetaphosphate remains in solution and the other phosphates are precipitated as barium salts. In these separation methods, the cross-contamination between a precipitating component and a non-precipitating component cannot be completely avoided.

The second is fractional precipitation of condensed phosphates with an organic solvent which is miscible with water. For instance, when a mixed solution of a series of linear phosphates is treated with acetone, higher polymers are precipitated as oily substances and lower polymers remain in solution. By successive treatments with acetone, the mixture of linear phosphates can be fractionated into several groups.

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Although these precipitation methods are very useful for special purposes, in general the resolution of a given couple of species is poor. On the other hand, the various methods based on chromatographic techniques give a better resolution for the separation of individual phosphorus oxoacids. Moreover, it is often possible to separate a number of phosphorus oxoacids from one another by means of only one chromatographic run.

Chromatography for phosphorus oxoacids will be discussed in the following order; paper and thin-layer chromatography, paper electrophoresis, ion-exchange chromatography, and gel chromatography. Although paper electrophoresis is not involved in the category of chromatography in a narrow sense, it is included in this discussion for convenience.

Table 3. Classification of phosphorus oxoacids

A: Oxidation number of five
  1. Linear phosphates: $P_1, P_2, P_3 \ldots$
  2. Cyclic phosphates: $P_{3m}, P_{4m}, P_{5m} \ldots$
  3. Branched phosphates

B: Lower oxidation number
$$\overset{1}{P},\ \overset{3}{P},\ \overset{2}{P}-P,\ \overset{2}{P}-\overset{2}{P},\ \overset{4}{P}-\overset{4}{P},\ \overset{4}{P}-\overset{4}{P},\ \overset{3}{P}-\overset{4}{P}-P,\ (-\overset{3}{P}-)_6-\text{ring},$$
$$\overset{3}{P}-O-P,\ \overset{3}{P}-O-\overset{3}{P},\ \overset{5}{P}-O-\overset{3}{P},\ \overset{4}{P}-O-\overset{4}{P},\ \overset{4}{P}-\overset{4}{P}-O-\overset{4}{P}-\overset{4}{P},$$
$$(-\overset{4}{P}-\overset{4}{P}-O-)_2-\text{ring}$$

C: Higher oxidation number
$(HO)_2P(O)OOH$
$(HO)_2P(O)OOP(O)(OH)_2$

PAPER AND THIN-LAYER CHROMATOGRAPHY

For the separation of phosphorus oxoacids by thin-layer chromatography, cellulose powder is usually employed, though some investigations by the use of silica gel, corn starch, diatomaceous earth, or anion exchangers have been reported[4]. Therefore, paper and thin-layer chromatography are discussed together in this section. For paper and thin-layer chromatography in which cellulose is used as a supporting material, sample solutes are in general separated by the mechanism based on their partition between two phases. As for paper chromatography of phosphorus oxoacids this has been verified by the comparison of the batch equilibrium data between two liquid phases, namely aqueous and organic, with the paper chromatographic data[5]. For these cases the stationary phase is considered to be composed of a mixture of cellulose and the solvent which contains more water than the developing solvent, i.e. the mobile phase. In some cases adsorption must be considered as a side effect. Impurities in filter paper such as calcium and iron may adsorb phosphorus oxoanions. The direct interaction of sample solutes with cellulose cannot entirely be ignored in some solvent systems like a basic benzyl alcohol system[5].

It is well known that filter paper can act as a cation-exchange medium. Although its ion-exchange capacity is extremely low, i.e. about 1 to 10 µeq/g

dry cellulose, this property plays an important role in some cases. From this point of view, Rommel and Keller[6] have explained the fact that sodium orthophosphate gives two spots on a paper chromatogram developed under certain conditions.

For the separation of phosphorus oxoacids a number of developing solvents have been proposed. These solvents are usually divided into two groups, namely acidic and basic solvents. Many data for R_f values of phosphorus oxoacids obtained by paper and thin-layer chromatography with these acidic and basic solvents indicate the following tendencies. As for a homologous series, the R_f values usually decrease with increase of the degree of polymerization. This can be explained by the fact that a lower polymer is more soluble in a mobile phase, which is less polar than a stationary phase, in comparison with a higher polymer. The R_f values of a linear phosphorus oxoacid obtained with acidic solvents are usually higher than those obtained with basic solvents. On the contrary, the R_f values of a cyclic phosphorus oxoacid obtained with acidic solvents are usually lower than those obtained with basic solvents. These tendencies are closely connected with the difference between the dissociation constants of linear and cyclic phosphorus oxoacids and with the difference of water contents in the acidic and basic solvents.

Iida and Yamabe[7] have found that the R_m values defined by equation (1)

$$R_m = \log \{(1/R_f) - 1\} \tag{1}$$

of a homologous series of linear or cyclic phosphates in thin-layer chromatography have a linear relationship with the number of their middle PO_4 units. Since there is a linear relationship between R_m and Gibbs free energy, these results demonstrate the additivity of Gibbs free energy necessary to transport one mole of each middle PO_4 unit of linear or cyclic phosphates from the stationary phase to the mobile phase.

Characteristics for separation by paper and thin-layer chromatography are a small sample-amount necessary for analysis, the simplicity and rapidity of the procedure, and the facility of simultaneous development of a sample and a standard. On account of these characteristics paper and thin-layer chromatography are mainly used for the purpose of identification. However, it is also possible to determine the amounts of sample components separated as spots or bands on chromatograms.

The most striking advantage of paper and thin-layer chromatography over column chromatography is the capability for two-dimensional chromatography. The examples for the analysis of complicated mixtures which cannot be separated unless two-dimensional chromatography is employed will be described below.

Paper and thin-layer chromatography are usually applied to the separation of the linear and cyclic phosphates with the degrees of polymerization lower than ten. However, when the development is carried out very slowly, a series of linear phosphates with degrees of polymerization from one to fourteen can be separated as shown in *Figure 1*[8, 9]. For the chromatographic solvents generally employed, linear phosphates with degrees of polymerization higher than ten do not move from the origin under normal development conditions. However, when Ebel's solvent, which is one of the most frequently
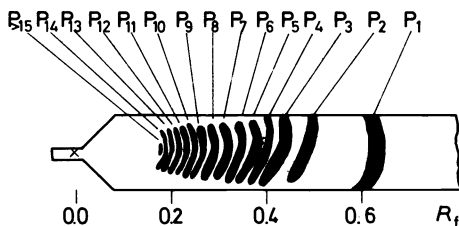
Figure 1. Paper chromatogram for a series of linear phosphates: sample—a glass of the Na_2SiO_3-$NaPO_3$ system (P/Si = 20); solvent—58 cm^3 of acetone, 25 cm^3 of 20% trichloroacetic acid, and 17 cm^3 of water; paper—Toyo-roshi No. 51A, 60 × 8 cm; 10° C; 69 h; solvent front—52.5 cm; reproduced by permission of the Chemical Society of Japan[9].

employed acidic solvents and composed of isopropanol, trichloroacetic acid, ammonia, and water, is diluted with water, the highly polymerized linear phosphates move appreciably from the origin[10]. This technique is useful for qualitatively estimating the size distribution of a mixture of highly polymerized linear phosphates.
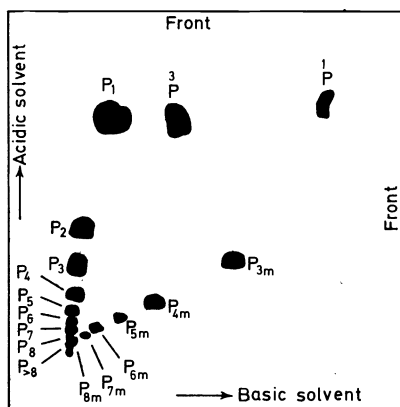
Figure 2. Two-dimensional thin-layer chromatogram for a complicated mixture of phosphinate, phosphonate and two series of linear and cyclic phosphates: thin layer—cellulose MN 300; acidic solvent (vol. %)—ethanol (10), n-propanol (50), water (40), and trichloroacetic acid (1 g/100 cm^3); basic solvent (vol. %)—ethanol (60), water (35), concentrated aqueous ammonia (5), and trichloroacetic acid (1.5 g/100 cm^3); reproduced by permission of Springer–Verlag[12].

By two-dimensional paper[11] or thin-layer chromatography[12] the linear phosphates with degrees of polymerization from one to eight and cyclic phosphates with those from three to eight can be completely separated from one another (*Figure 2*). This separation cannot be achieved by any other chromatrographic techniques.

Twelve kinds of phosphorus oxoacids with lower oxidation numbers have so far been isolated as alkali or ammonium salts which are stable in neutral

aqueous solution. Paper chromatographic behaviour of all these phosphorus oxoacids has been studied by means of a pair of acidic and basic solvents (*Figure 3* [13]). The separation of lower phosphorus oxoacids by two-dimensional paper chromatography has also been reported[14]. Recently a very complicated sample containing homologous series of linear and cyclic phosphates and monofluoro- and difluoro- linear phosphates was successfully separated by two-dimensional paper chromatography[15].

PAPER ELECTROPHORESIS

In paper electrophoresis the migration of a sample solute in an electric field is the major factor for the separation mechanism. Filter paper acts in general only as a supporter of solution. When the very dilute solution of a supporting electrolyte is used, however, a sample solute might be adsorbed by filter paper. In some cases a certain solute reacts with a supporting electrolyte to produce a precipitate. In such a case, the solute remains at the starting line. An example for this case will be shown later.

For paper electrophoresis Jokl[16] has derived equation (2)

$$u = (az/m^{\frac{1}{2}}) + bz \qquad (2)$$

based on theoretical considerations and experimental results, where u is the zone mobility, z and m are the charge number and the molecular weight of the migrating species, and a and b are constants. The charge number, z, of a given weak acid such as many phosphorus oxoacids is dependent on the pH of a solution. Therefore, the zone mobility, u, is a function of the pH and the molecular weight. If a supporting electrolyte reacts with phosphorus oxoacids to form complexes, these reactions should be taken into consideration.
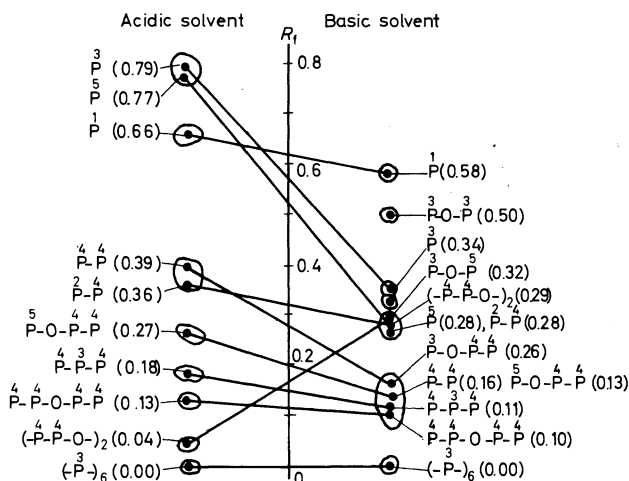

Figure 3. The R_f values of some lower phosphorus oxoanions obtained by ascending paper chromatography: paper—Toyo-roshi No. 51A; acidic solvent—325 cm³ of acetone, 1.75 cm³ of 28% aqueous ammonia, 150 cm³ of water, and 25 g of trichloroacetic acid; basic solvent—190 cm³ of ethanol, 150 cm³ of isobutanol, 2.7 cm³ of 28% aqueous ammonia, and 225.3 cm³ of water; 5–7°C; reproduced by permission of Elsevier Publishing Company[13].
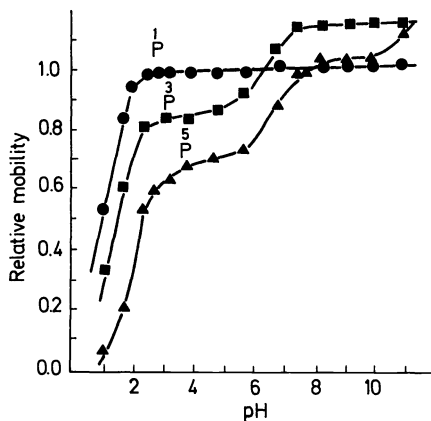
Figure 4. The observed zone mobility/pH curves for phosphorus oxoacids: paper—Toyo-roshi No. 50, 2 × 40 cm; supporting electrolyte solution—mixtures of hydrochloric acid, ammonium acetate and ammonia with pH 1 to 11 and ionic strength of 0.1; 500 V/34 cm; 20 ± 1°C; 1 h; reproduced by permission of Elsevier Publishing Company[17].

According to the investigation by Kiso and his co-workers[17], the relative mobilities of phosphinic (hypophosphorous), phosphonic (phosphorous), and orthophosphoric acids vary regularly with the change of pH of the supporting electrolyte solution as shown in *Figure 4*. Since phosphinic acid shows the dissociation of only one step, it gives a constant mobility in a wide range of pH. On the other hand, since phosphonic and orthophosphoric acids show the dissociation of two steps and three steps, these acids give the two-step and three-step curves in a mobility versus pH diagram, respectively.

The separation of phosphorus oxoacids by paper electrophoresis has so far frequently been employed for studies on the hot atom chemistry of ^{32}P[18,19]. For this purpose a mixed solution of zinc acetate and lactic acid has been recommended as a supporting electrolyte for the separation of very complicated mixtures of ^{32}P-labelled phosphorus oxoacids produced in solid inorganic phosphates by the n–γ reaction. As has been described in the early part of this paper, pyrophosphate precipitates as zinc pyrophosphate by the addition of zinc ions in a weakly acidic solution. By the use of this supporting electrolyte, therefore, pyrophosphate ions remain at the starting line, being clearly separated from many other phosphorus oxoanions.

Paper electrophoretic behaviour of twelve kinds of phosphorus oxoanions with lower oxidation numbers and of ortho-, pyro-, and triphosphate anions has been systematically investigated by using mixed solutions of zinc acetate and lactic acid with various compositions as supporting electrolytes (*Figures 5 and 6*)[20]. Under the conditions given in *Figure 5* P$_4$—P$_3$—P$_4$ also precipitates at the starting line.

Paper electrophoresis is often employed for the purpose of identification like paper and thin-layer chromatography. However, in the cases such as the
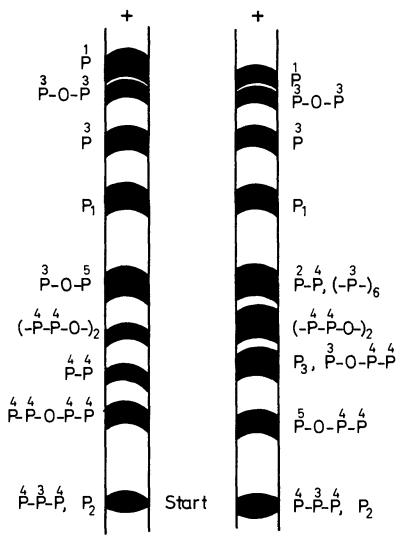
Figure 5. Chromatograms for phosphorus oxoacids obtained by paper electrophoresis: paper—Toyo-roshi No. 51A, 2 × 60 cm; supporting electrolyte solution—0.03 M zinc acetate–0.04 M lactic acid with pH 4.2 and ionic strength of 0.1; 28 V/cm; 10°C; 2 h; reproduced by permission of the Japan Society for Analytical Chemistry[20].

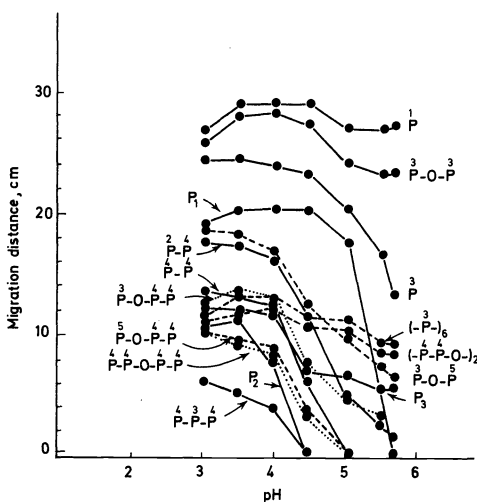studies of hot atom chemistry, the purpose of quantitative analysis can also be achieved by measuring ^{32}P activities of the spots separated on a sheet of paper.

Figure 6. The pH dependence of the migration distances of phosphorus oxoacids in paper electrophoresis: paper—Toyo-roshi No. 51A, 2 × 60 cm; supporting electrolyte solution—0.03 M zinc acetate–0.1–1 M lactic acid with ionic strength of 0.1; 28 V/cm; 10°C; 2 h; reproduced by permission of the Japan Society for Analytical Chemistry[20].

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ION-EXCHANGE CHROMATOGRAPHY

The major factor for the separation mechanism in ion-exchange chromatography is, of course, the ion exchange of a solute between an ion-exchanger phase and a solution phase. However, for the behaviour of highly polymerized phosphates, the molecular-sieve effect must be taken into account[21].

The separation methods of phosphorus oxoacid by ion-exchange chromatography have been developed by the efforts of many investigators. In the early stage of this development Rieman and his co-workers presented an important paper[22] with respect to a fundamental investigation of ion-exchange chromatography, dealing with linear and cyclic phosphates with low degrees of polymerization as examples. Equations were developed to describe the elution curves of various phosphoric acids as functions of the pH and concentrations of the eluent solutions. This work has advanced the theory of ion-exchange chromatography and pointed the way to the development of an accurate procedure for the analysis of mixtures of the polymeric phosphates.

For the separation of phosphorus oxoacids, anion-exchange resins such as Dowex-1 and Amberlite IRA-400 are usually employed. These resins are strong-base anion exchangers composed of quaternary-ammonium derivatives of crosslinked polystyrene. As for eluents, sodium or potassium chloride are often used.

The most important parameter in ion-exchange chromatographic separation is a distribution ratio, D,

$$D = C_r/C_s \qquad (3)$$

where C_r and C_s are the concentrations of a given solute in the resin phase and solution phase, respectively. When a resin and an eluent agent are fixed, the distribution ratio of a phosphorus oxoacid is described as a function of the pH and concentration of the eluent solution. By checking the distribution ratios of phosphorus oxoacids contained in a given sample, the optimum conditions for the separation are decided.

Ion-exchange chromatography can be employed not only for identification but also for quantitative analysis. For the latter purpose, ion-exchange chromatography has advantages over paper and thin-layer chromatography and paper electrophoresis, because of the larger amount of a sample to be treated in the former. Moreover, by the use of a large column of ion-exchanger, it is also possible to accomplish a preparative work.

Orthophosphate and a few condensed phosphates with relatively low degrees of polymerization such as pyro- and triphosphate can be separated on an ion-exchange resin column by the stepwise-elution technique which is based on the stepwise increase of an eluent concentration. The analysis of a sample composed of a number of phosphorus oxoacids can be efficiently accomplished by a gradient elution technique. In many cases an exponentially increasing gradient of eluent concentration is employed. Generally applicable equations for the calculation of elution peak positions in the exponential gradient elution of ions whose charges are not necessarily integral have been derived[23-25]. A series of linear phosphates from ortho- to heptadecaphos-

phate was separated on a Dowex 1X4 resin column by eluting with potassium chloride solution of exponentially increasing concentration (*Figure 7*)[25].

As has been described before, the pH of an eluent solution plays an important role for the separation of phosphorus oxoacids. The pH depen-
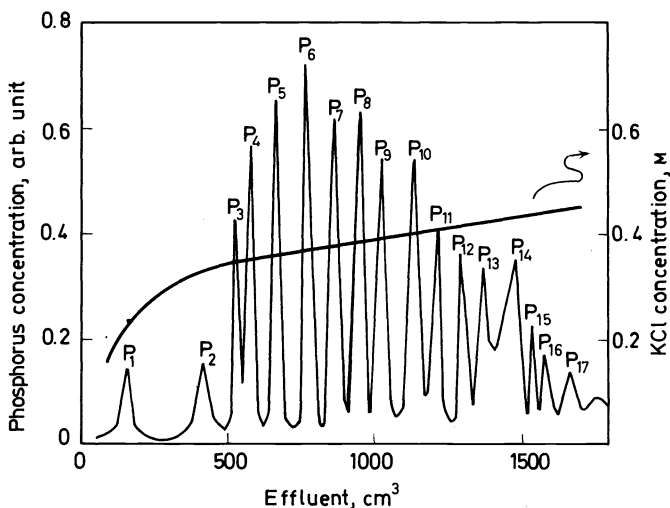
Figure 7. Elution curves for a series of linear phosphates obtained by ion-exchange chromatography: sample—a mixture of P_1, P_2, and glassy sodium polyphosphate with an average degree of polymerization of 10; anion-exchange resin—Dowex 1 X4, chloride form, 1.2 × 69 cm; eluent— potassium chloride solution, pH 8.0; room temperature; reproduced by permission of Elsevier Publishing Company[25].

dence of anion-exchange chromatographic separation of tri-, tetra-, and pentaphosphate anions has been investigated in detail[26]. As has been shown in *Figure 7*, the difference between the elution positions of tri- and tetra-phosphate is remarkably smaller than those between the elution positions of other adjacent pairs.

The chromatographic separation of the linear phosphates will be discussed in terms of a separation factor, α_a^b, which is defined by

$$\alpha_a^b = D_b/D_a \tag{4}$$

where D_a and D_b represent the distribution ratios of phosphate species a and b, respectively. α_3^4 and α_4^5 were calculated from the batch distribution ratios of tri-, tetra-, and pentaphosphate. The pH dependence of these separation factors is shown in *Figure 8*. All the curves for α_3^4 give minimum values near pH 7 for all chloride concentrations. On the other hand, the curves for α_4^5 exhibit a tendency similar to those for α_3^4 at low chloride concentrations, but those at high chloride concentrations seem to have maximum values. This suggests that the separation of tri- and tetraphosphate will be improved if the pH of eluent is kept apart from pH 7, but the separation of tetra- and pentaphosphate will not be appreciably improved by changing
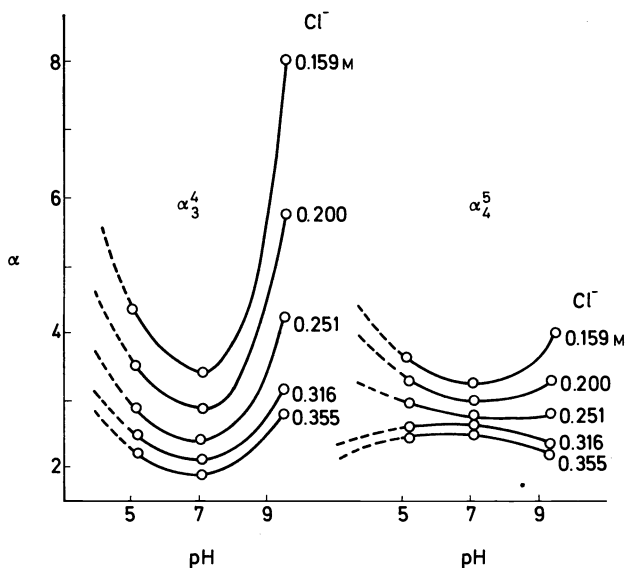
Figure 8. The pH dependence of α_3^4 and α_4^5 at various chloride concentrations: anion-exchange resin—Dowex 1X4, chloride form; solutions—0.16–0.36 M potassium chloride of pH 5.2, 7.1, and 9.5; 20°C; 2 h; reproduced by permission of the Chemical Society of Japan[26].
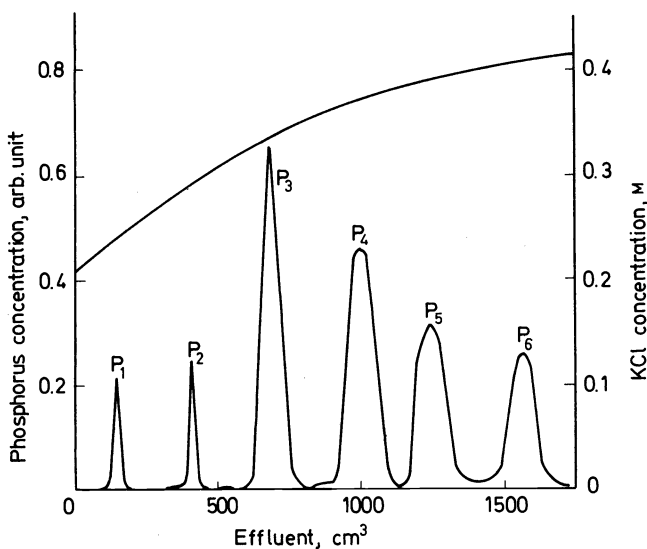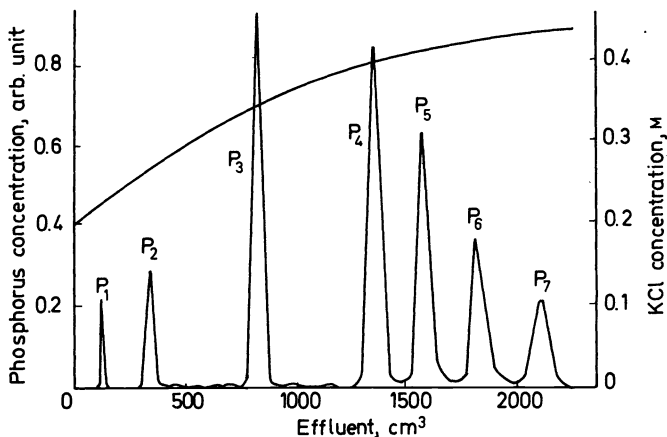

Figure 9. The elution curve for a series of linear phosphates obtained by ion-exchange chromatography: anion-exchange resin—Dowex 1X4, chloride form, 1.24 × 66.5 cm; eluent—potassium chloride solution of pH 4.5; 20°C; reproduced by permission of the Chemical Society of Japan[26].

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Figure 10. The elution curve for a series of linear phosphates obtained by ion-exchange chromatography: anion-exchange resin—Dowex 1X4, chloride form, 1.24 × 66.5 cm; eluent—potassium chloride solution of pH 3.0; 20°C; reproduced by permission of the Chemical Society of Japan[26].
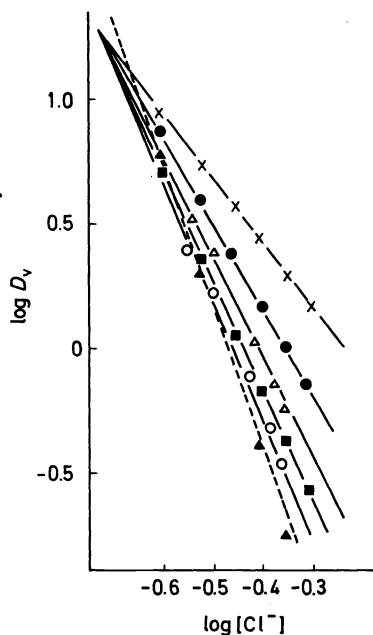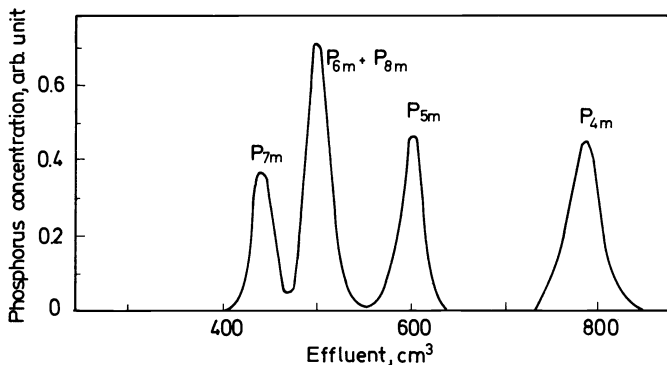

Figure 11. The plots of log D_v versus log $[Cl^-]$ for a series of cyclic phosphates: anion-exchange gel—QAE-Sephadex A-25, chloride form; solutions—0.25–0.50 M potassium chloride of pH 5.2; room temperature; × — ×, P_{3m}; ● — ●, P_{4m}; △ — △, P_{5m}; ■ — ■, P_{6m}; ○ — ○, P_{7m}; ▲ – – – ▲, P_{8m}; reproduced by permission of Elsevier Publishing Company[27].

the pH of eluent in the high chloride concentration region where these two phosphates are practically eluted out. This was confirmed by the chromatographic runs at the different pH values of 4.5 and 3.0, which are shown in *Figures 9* and *10*.

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For the separation of a series of cyclic phosphates from trimeta- to octametaphosphate, QAE-Sephadex A-25, which is an anion-exchange dextran gel, has been successfully employed[27]. Distribution ratios of the cyclic phosphates were measured in order to determine the eluent concentration for the best separation. Potassium chloride was used as an eluent. The plots of the logarithms of the distribution ratios, D_v, versus those of the eluent concentrations are shown in *Figure 11*.

Figure 12. The elution curve for a series of cyclic phosphates obtained by ion-exchange chromatography: anion-exchange gel—QAE-Sephadex A-25, chloride form, 1.5 × 88 cm; eluent—0.30 M potassium chloride of pH 5.2; room temperature; reproduced by permission of Elsevier Publishing Company[27].
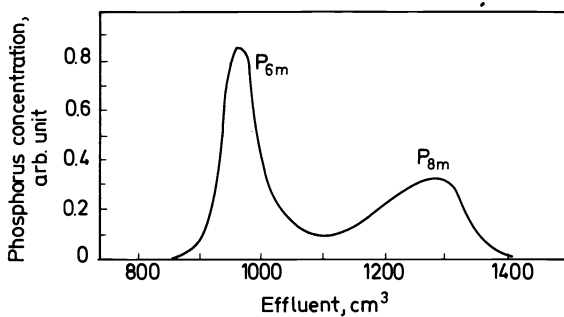

Figure 13. The elution curve for hexameta- and octametaphosphate obtained by ion-exchange chromatography: anion-exchange gel—QAE-Sephadex A-25, chloride form, 1.5 × 88 cm; eluent—0.25 M potassium chloride of pH 5.2; room temperature; reproduced by permission of Elsevier Publishing Company[27].

In general, the eluent concentration should be chosen so that a ratio of the distribution ratios of a given couple in the sample 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 consideration, 0.30 M potassium chloride was chosen as an eluent (log 0.30 = −0.52). The elution curve for the mixture of cyclic phosphates is shown in *Figure 12*. If trimetaphosphate is present in a sample, it is eluted at about 1100 cm³ of effluent under the conditions

given in *Figure 12*. 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 as can be expected from the data of *Figure 11*. However, the separation of these two species can be made with 0.25 M potassium chloride as shown in *Figure 13*.

As an example of the application of ion-exchange chromatography, the studies on hot atom chemistry of ^{32}P will be mentioned. Previous investigations have indicated that a number of kinds of ^{32}P-labelled phosphorus oxoanions are formed by recoil reactions in neutron-irradiated solid inorganic orthophosphates. Among these ^{32}P-labelled products linear phosphates have a great significance in relation to the mechanisms of recoil reactions in irradiated orthophosphates, because the formation of linear phosphates with higher degrees of polymerization would indicate much fragmentation in the reaction zone. Although the formation of ^{32}P-labelled linear phosphates in irradiated orthophosphates had already been demonstrated by several workers[28], the molecular size and distribution of the linear phosphates had not been clearly established. We have applied anion-exchange chromatography for the analysis of ^{32}P-labelled products in neutron-irradiated solid inorganic orthophosphates[29-31]. Since the ^{32}P-labelled products are a complicated mixture of various phosphorus oxoanions, the gradient of eluent concentration should be precisely controlled during a chromatographic run. *Figure 14* shows the elution curve for a mixture of authentic samples of fourteen kinds of phosphorus oxoacids. *Figure 15* indicates the elution curve for a neutron-irradiated potassium dihydrogen orthophosphate.

By such studies it has been established that a series of ^{32}P-labelled linear phosphates from pyro- to penta- or higher polyphosphates is formed in irradiated potassium or ammonium dihydrogen orthophosphate. This work suggests that the size of the reaction zone produced by recoil atoms in solid orthophosphates is not so small that it contains only two or three phosphorus atoms, but is a considerably larger one containing five or more phosphorus atoms. It is also interesting and important to know which position in a linear
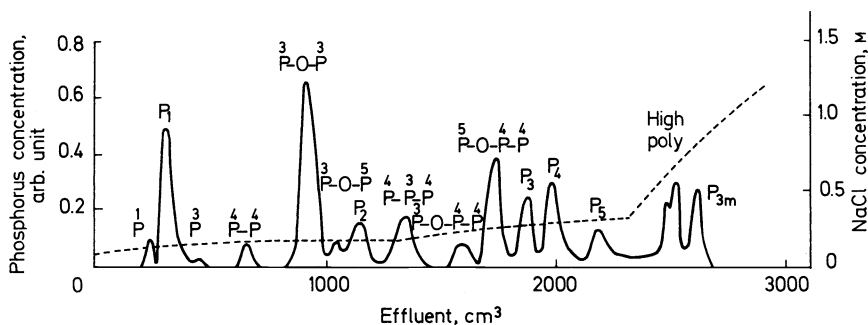
Figure 14. The elution curve for a complicated mixture of phosphorus oxoacids obtained by ion-exchange chromatography: anion-exchange resin—Dowex 1X8, chloride form, 1.3 × 63 cm; eluent—potassium chloride solution of pH 6.8; room temperature; reproduced by permission of Pergamon Press Limited[31].
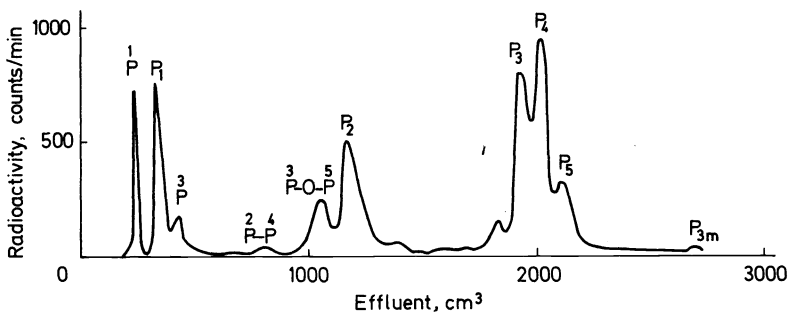
Figure 15. The elution curve for a neutron-irradiated potassium dihydrogen orthophosphate dissolved in a carrier solution: the elution conditions—the same as those described in *Figure 14*; reproduced by permission of Pergamon Press Limited[31].
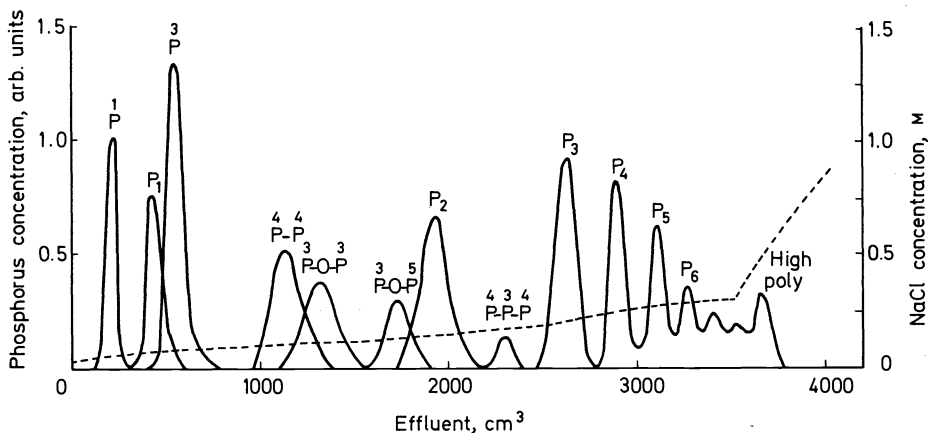

Figure 16. The elution curve for a complicated mixture of phosphorus oxoacids obtained by ion-exchange chromatography[32]: anion-exchange resin—BioRad AG 1–X8, chloride form, 1.3 × 68 cm; eluent—sodium chloride solution of pH7, containing 0.005 M EDTA; room temperature.

phosphate molecule is occupied by a ^{32}P atom, i.e. the intramolecular distribution of ^{32}P atoms in a linear phosphate molecule. At present we are extending our studies in this direction.

In the course of studies on the separation of ^{32}P recoil species produced in irradiated alkali orthophosphates by anion-exchange chromatography, it was observed that the addition of some phosphorus oxoanions as carriers to a solution of the irradiated material gives a quite different elution pattern of ^{32}P-labelled species from that obtained in the absence of the carriers. We call these phenomena the carrier effects. We presume that the carrier effects may be due to some metal ions as impurities contained in a target and reagents used for the chromatographic separation. Recently we have employed an

eluent solution containing sodium chloride and sodium ethylenediaminetetra-acetate in order to avoid the interference due to iron (III) ions present in various reagents and an anion-exchange resin. An example of elution curves obtained by this method is shown in *Figure 16*[32].

GEL CHROMATOGRAPHY

In our laboratory the chromatographic behaviour of phosphorus oxoacids has been investigated by the use of various kinds of Sephadex gels, which are crosslinked dextran gels[33-35]. Some of the results will be mentioned.

The major factor for the separation mechanism in gel chromatography is the molecular-sieve effect, i.e. the steric-exclusion effect. In some cases, however, ion exclusion and adsorption should be taken into consideration. When pure water is used as an eluent for a column of Sephadex, phosphorus oxoanions are excluded from a gel phase by the ion-exclusion effect due to the repulsion between the phosphorus oxoanions and fixed negative charges of the gel matrix, which are believed to be carboxyl groups. Since the amount of the fixed negative charges is very small, the ion-exclusion effect can be eliminated by the addition of a supporting electrolyte such as potassium chloride to an eluent. On the other hand, borate and perchlorate ions are strongly adsorbed by a dextran gel.
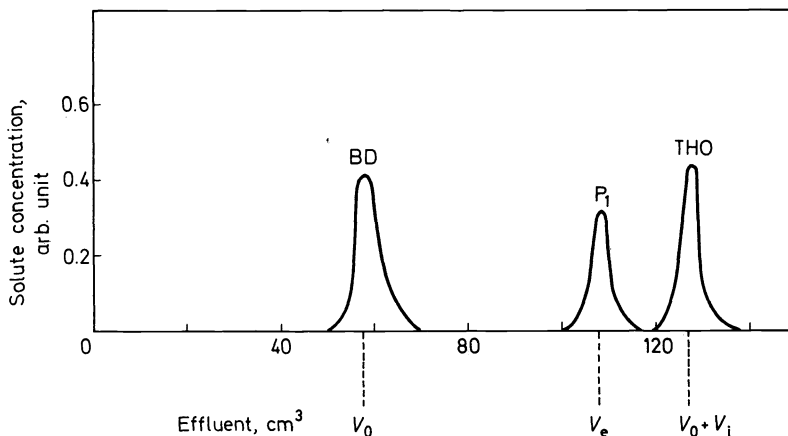
Figure 17. Standardization of columns in gel chromatography: gel— Sephadex G-25, 1.5 × 90 cm; eluent—0.1 M potassium chloride; room temperature; BD = Blue Dextran 2000, THO = tritiated water.

In gel chromatography the standardization of columns gives an advantage because results obtained with different columns can readily be compared with one another (*Figure 17*). Blue Dextran 2000, which is a blue derivative of dextran with a molecular weight of 2×10^6, or potassium Kurrol's salt was used as a standard substance with a distribution coefficient, K_d, of zero.

Tritiated water, THO, was used as a standard substance with K_d of unity. For a given sample solute, its K_d value can be calculated by equation (5),

$$V_e = V_o + K_d \times V_i \tag{5}$$

where V_e is the elution volume, V_o the void volume outside the gel particles, and V_i the internal volume within the gel particles. V_o and V_i can be obtained by the chromatographic runs for the standard substances mentioned above.

Figure 18 shows the effect of gel porosity on the elution curves of ortho-, pyro-, and triphosphate. With the increase of the G-number, i.e. increase of the pore size, the K_d values of these anions increase as expected. For the
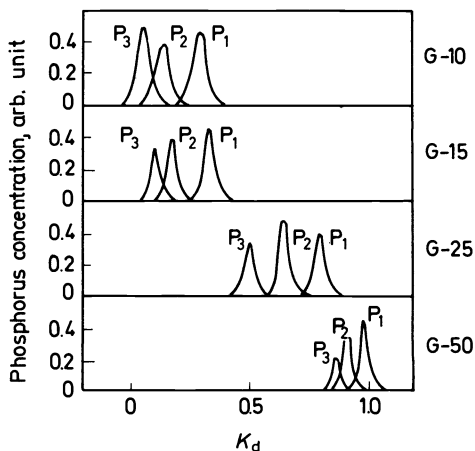

Figure 18. The effect of gel porosity on the elution curves for P_1, P_2, and P_3 in gel chromatography: gel—Sephadex G-10, G-15, G-25, and G-50; eluent—0.1 M potassium chloride; room temperature; reproduced by permission of Elsevier Publishing Company[34].
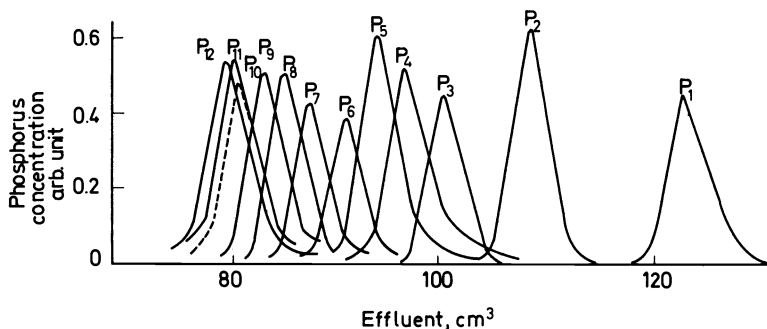

Figure 19. The elution curves for a series of linear phosphates obtained by gel chromatography: elution conditions—the same as those described in *Figure 17*; reproduced by permission of Elsevier Publishing Company[35].

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separation of these three acids Sephadex G-25 is most suitable. The elution curves for the linear phosphates with degrees of polymerization of one to twelve on a Sephadex G-25 column are shown in *Figure 19*.

We have tried to estimate the effective sizes of phosphorus oxoanions. For this purpose, another distribution coefficient, K_{av}, must be introduced. K_{av} is defined by equation (6),

$$V_e = V_o + K_{av}(V_t - V_o) \tag{6}$$

where V_t is the total volume of the gel bed. Laurent and Killander[36] have presented equation (7),

$$(-\log K_{av})^{\frac{1}{2}} = A \times R_s + B \tag{7}$$

where R_s is the radius of a sample solute molecule and A and B are constants. If one uses proper reference substances with known R_s values, one can estimate the effective sizes of phosphorus oxoanions.

All of the data thus obtained are summarized in *Figure 20*, in which the values of K_{av} and R_s are shown in the different scales. *Figure 20* indicates the possibility for group separation of the phosphorus oxoanions which contain one to four phosphorus atoms. These ions can be separated into three groups on a Sephadex G-25 column. The first group is the oxoanions having one phosphorus atom. The second group is those having two phosphorus atoms and $\overset{4}{P}$—$\overset{3}{P}$—$\overset{4}{P}$. The third group is those having three and four phosphorus atoms.
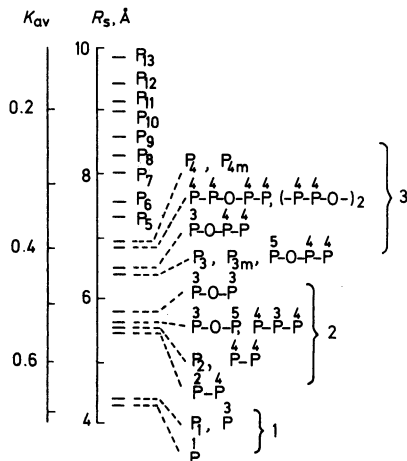
Figure 20. Estimation of the effective sizes of phosphorus oxoanions in aqueous solution by gel chromatography[34]: elution conditions—the same as those described in *Figure 17*.

In gel chromatography the linear relationship between K_d or K_{av} values of solute molecules and the logarithms of their molecular weights or degrees of polymerization is often observed. *Figure 21* indicates that there is a similar relationship between the K_{av} values of a series of linear phosphates
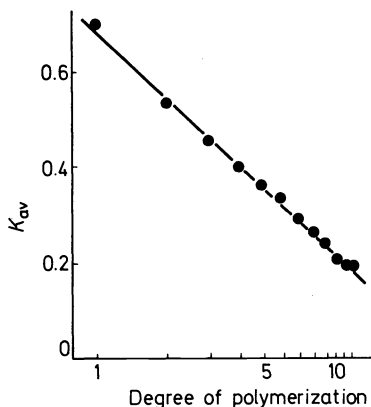
Figure 21. Relationship between the K_{av} values of a series of linear phosphates and the logarithms of their degrees of polymerization: elution conditions—the same as those described in *Figure 17*; reproduced by permission of Elsevier Publishing Company[35].

and the logarithms of their degrees of polymerization. Although this relationship is quite empirical, it is useful to estimate molecular weights or distribution of linear phosphates in their mixture.

In order to test the applicability of the above relationship, average degrees of polymerization of some sodium polyphosphate mixtures were determined by gel chromatography and by the usual pH titration method. Good agreement was observed between the results obtained by both methods (*Table 4*).

Table 4. Average degrees of polymerization of sodium polyphosphate fractions determined by gel chromatography and pH titration
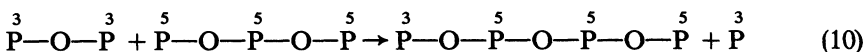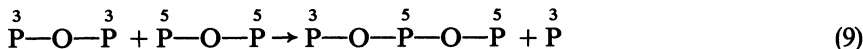Gel, Sephadex G–25; bed volume, 150 cm^3; eluent, 0.1 M KCl (pH 7.0).

Gel chromatography	3.2	8.4	9.3	9.5	12.0	14.0
pH titration	3.0	8.2	9.1	10.1	11.0	13.1

As an example of the application of gel chromatography to oxoacid polymers of phosphorus, we will refer to the substitution reactions between diphosphinate and polyphosphate[37].

It is well known that orthophosphate, $\overset{5}{P}$, reacts with diphosphinate, $\overset{3}{P}$—O—$\overset{3}{P}$, to produce isohypophosphate, $\overset{3}{P}$—O—$\overset{5}{P}$, according to equation (8)[38]. Analogous substitution reactions may be expected to proceed according to equations (9) and (10) when pyrophosphate, $\overset{5}{P}$—O—$\overset{5}{P}$, and triphosphate, $\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$, are used instead of $\overset{5}{P}$ in equation (8).

$$\overset{3}{P}\text{—O—}\overset{3}{P} + \overset{5}{P} \rightarrow \overset{3}{P}\text{—O—}\overset{5}{P} + \overset{3}{P} \tag{8}$$

$$\overset{3}{P}-O-\overset{3}{P} + \overset{5}{P}-O-\overset{5}{P} \rightarrow \overset{3}{P}-O-\overset{5}{P}-O-\overset{5}{P} + \overset{3}{P} \qquad (9)$$

$$\overset{3}{P}-O-\overset{3}{P} + \overset{5}{P}-O-\overset{5}{P}-O-\overset{5}{P} \rightarrow \overset{3}{P}-O-\overset{5}{P}-O-\overset{5}{P}-O-\overset{5}{P} + \overset{3}{P} \qquad (10)$$

The formation of $\overset{3}{P}-O-\overset{5}{P}-O-\overset{5}{P}$ was first suggested by Blaser and Worms[38] and thin-layer chromatographic evidence for the presence of such a new species has been reported[14]. There had been no information on an attempt to investigate the formation of $\overset{3}{P}-O-\overset{5}{P}-O-\overset{5}{P}-O-\overset{5}{P}$. Then, this work was undertaken to characterize the gel chromatographic behaviour of the reaction products in equations (9) and (10) with a view to obtaining positive evidence for the formation of such new or hypothetical compounds as $\overset{3}{P}-O-\overset{5}{P}-O-\overset{5}{P}$ and $\overset{3}{P}-O-\overset{5}{P}-O-\overset{5}{P}-O-\overset{5}{P}$. The gel chromatographic technique using a Sephadex G-25 column was employed in this work.

A mixture of equal volumes of 0.25 M $\overset{3}{P}-O-\overset{3}{P}$ (disodium salt) and 0.25 M $\overset{5}{P}-O-\overset{5}{P}$ (tetrasodium salt) was allowed to react at 20°C. At proper time intervals 1 cm³ of the test solution was withdrawn and eluted on a Sephadex G-25 column (1.5 × 85 cm) by the use of a 0.1 M sodium chloride solution as an eluent. The amount of phosphorus in each fraction was determined by colorimetry.
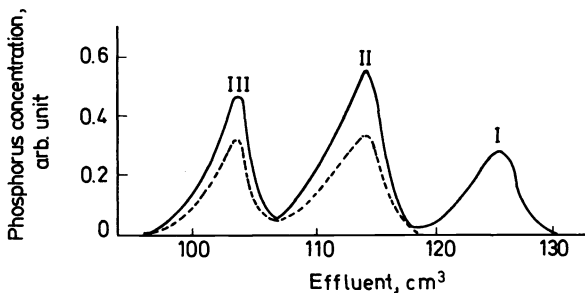

Figure 22. The elution curve for the reaction products between $\overset{3}{P}-O-\overset{3}{P}$ and $\overset{5}{P}-O-\overset{5}{P}$ obtained by gel chromatography: gel—Sephadex G-25, 1.5 × 85 cm; eluent—0.1 M sodium chloride; reaction time—9 days; solid line—$\overset{3}{P}$-unit + $\overset{5}{P}$-unit; broken line—$\overset{5}{P}$-unit; reproduced by permission of Pergamon Press Limited[37].

Figure 22 shows an elution curve for the reaction products after nine days. Peaks I, II and III correspond to the elution positions of monomer, dimer and trimer, respectively. As shown in *Figure 23*, the relative amounts of peaks I and III increased with time at the expense of peak II. By differential analysis of phosphorus of oxidation numbers 3 and 5, it was found that peak I contained only $\overset{3}{P}$ and peak III was composed of $\overset{3}{P}$- and $\overset{5}{P}$-units. The ratio
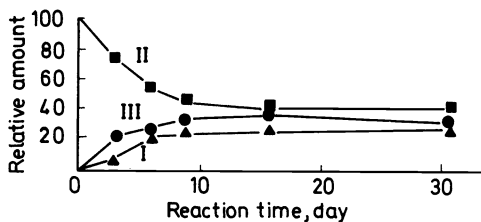
Figure 23. The change of relative amounts of total phosphorus in peaks I, II and III with reaction time; reproduced by permission of Pergamon Press Limited[37].
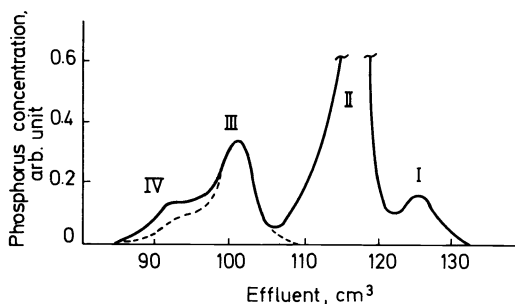

Figure 24. The elution curve for the reaction products between $\overset{3}{P}$—O—$\overset{3}{P}$ and $\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$ obtained by gel chromatography: elution conditions—the same as those described in Figure 22; reaction time—5 days; solid line—$\overset{3}{P}$-unit + $\overset{5}{P}$-unit; broken line—$\overset{5}{P}$-unit; reproduced by permission of Pergamon Press Limited[37].
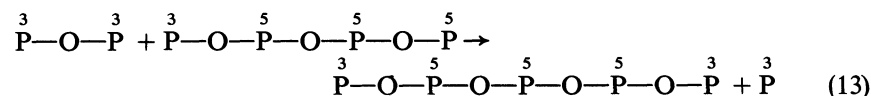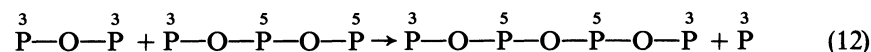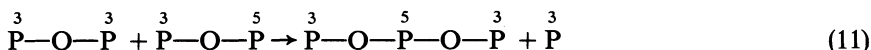
of $\overset{5}{P}$-unit to $\overset{3}{P}$-unit near the maximum of peak III was calculated to be 2.0 ± 0.2. which is expected from the composition of $\overset{3}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$.

In a similar manner, the reaction products between $\overset{3}{P}$—O—$\overset{3}{P}$ and $\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$ were separated by gel chromatography. As an example, the elution curve for the reaction products after five days is shown in Figure 24. Peaks II and III are due to the starting materials, i.e. $\overset{3}{P}$—O—$\overset{3}{P}$ and $\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$, respectively. Peak I is evidently due to $\overset{3}{P}$. Peak IV corresponds to the elution position of tetramer or higher polymers. However, since the ratio of $\overset{5}{P}$-unit to $\overset{3}{P}$-unit near and before the maximum of peak IV was estimated to be approximately three, the major component in peak IV may be $\overset{3}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{3}{P}$. The reaction products between $\overset{3}{P}$—O—$\overset{3}{P}$ and $\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$ were also separated by ion-exchange chromatography. In the elution curve for the reaction products after seven days a new peak appeared after the elution of the three known species, namely $\overset{3}{P}$, $\overset{3}{P}$—O—$\overset{3}{P}$,

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and $\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$. The ratio of $\overset{5}{P}$-unit to $\overset{3}{P}$-unit near the maximum of this new peak was almost equal to three. This fact also supports the conclusion that the major product is $\overset{3}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$.

Our present understanding for equations (8), (9) and (10) is based on the assumption that the reactions proceed according to S_N2 substitution in which $\overset{5}{P}$, $\overset{5}{P}$—O—$\overset{5}{P}$, and $\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$ act as nucleophilic agents to form $\overset{3}{P}$—O—$\overset{5}{P}$, $\overset{3}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$, and $\overset{3}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$, respectively. This understanding suggests the possibility that the polymers as the reaction products in equations (8), (9) and (10) furthermore react with $\overset{3}{P}$—O—$\overset{3}{P}$ to form polymers having $\overset{3}{P}$-units at both ends.

$$\overset{3}{P}\text{—O—}\overset{3}{P} + \overset{3}{P}\text{—O—}\overset{5}{P} \rightarrow \overset{3}{P}\text{—O—}\overset{5}{P}\text{—O—}\overset{3}{P} + \overset{5}{P} \tag{11}$$

$$\overset{3}{P}\text{—O—}\overset{3}{P} + \overset{3}{P}\text{—O—}\overset{5}{P}\text{—O—}\overset{5}{P} \rightarrow \overset{3}{P}\text{—O—}\overset{5}{P}\text{—O—}\overset{5}{P}\text{—O—}\overset{3}{P} + \overset{5}{P} \tag{12}$$

$$\overset{3}{P}\text{—O—}\overset{3}{P} + \overset{3}{P}\text{—O—}\overset{5}{P}\text{—O—}\overset{5}{P}\text{—O—}\overset{5}{P} \rightarrow$$
$$\overset{3}{P}\text{—O—}\overset{5}{P}\text{—O—}\overset{5}{P}\text{—O—}\overset{5}{P}\text{—O—}\overset{3}{P} + \overset{5}{P} \tag{13}$$

Up to the present we have got evidence that $\overset{3}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{3}{P}$ and $\overset{3}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{5}{P}$—O—$\overset{3}{P}$ are formed by equations (11) and (12), respectively.

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