Pyrophosphates as biocompatible packing materials for high-performance liquid chromatography

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ABSTRACT

Crystalline pyrophosphates of Mg, Ca, Sr, Mn and Zr were developed as new packing materials for high-performance liquid chromatography. They were synthesized as porous and rigid spheres based on a spray-pyrolysis method, followed by heat treatment. The pyrophosphate (PPi) packing materials thus obtained had high mechanical strength. All PPi columns tested showed similar chromatographic behaviours of basic proteins. In contrast, the chromatographic behaviour of acidic proteins, nucleotides and nucleic acids depended on the kind of PPi column: β-Ca$_2$P$_2$O$_7$, α-Sr$_2$P$_2$O$_7$ and Mn$_2$P$_2$O$_7$ columns showed similar chromatographic behaviour, whereas β-Mg$_2$P$_2$O$_7$ and ZrP$_2$O$_7$ columns showed no retention of most acidic proteins including phosphoproteins. Based on this property, a successful single-step separation of γ-globulin from other serum proteins by the β-Mg$_2$P$_2$O$_7$ column was achieved. The β-Mg$_2$P$_2$O$_7$ column could not retain nucleotides and the ZrP$_2$O$_7$ column could not retain nucleic acids.

INTRODUCTION

Various phosphates have been reported concerning their use as packing materials for high-performance liquid chromatography (HPLC). The chromatographic properties of calcium phosphates such as brushite [1,2], monetite and octacalcium phosphate [1] and calcium phosphate gel [3,4] have been studied. Hydroxyapatite (HAP) has been successfully used for the separation and purification of biomolecules [5,6]. Studies on zirconium phosphate ion exchangers dealt with crystalline material [7] and amorphous gels of variable composition [8].

A few attempts have been made to utilize a gel of magnesium pyrophosphate as a packing material [9,10]. However, the degree of hydration varied with ageing and the particles became smaller with time, so that the gel needed to be used immediately after preparation. In addition, the flow characteristics were too poor for its direct use in chromatographic columns.

No report has been published on the use of any sintered, crystalline pyrophosphate (PPi) packing materials in HPLC. Such pyrophosphates of alkaline earth metals are promising packing materials because of their high mechanical strength and stability at elevated pH and temperatures. This paper reports new preparations of crystalline pyrophosphates of calcium (CaPP; Ca$_2$P$_2$O$_7$), magnesium (MgPP; Mg$_2$P$_2$O$_7$), strontium (SrPP; Sr$_2$P$_2$O$_7$), manganese (MnPP; Mn$_2$P$_2$O$_7$) and zirconium (ZrPP; ZrP$_2$O$_7$) with a rigid spherical shape based on a spray-pyrolysis method. Their chromatographic properties were evaluated with proteins, nucleotides and nucleic acids.

EXPERIMENTAL

Materials

Lysozyme (chicken egg white) was obtained from Merck (Darmstadt, Germany). The follow-

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ing proteins were obtained from Sigma (St. Louis, MO, USA): cytochrome c (horse heart), ribonuclease A (bovine pancreas), α-chymotrypsinogen A (bovine pancreas), papain (papaya latex), α-chymotrypsin (bovine pancreas), myoglobin (horse skeletal muscle), haemoglobin (bovine blood), γ-globulin (human; prepared from Cohn fractions II and III), γ-globulin (bovine; prepared from Cohn fractions II and III), catalase (bovine liver), conalbumin (chicken egg white; iron poor), conalbumin (chicken egg white; iron complex), transferrin (human; apo type), transferrin (human; holo type), transferrin (bovine; apo type), transferrin (bovine; holo type), β-lactoglobulin A (bovine milk), β-lactoglobulin B (bovine milk), albumin (human serum), phosvitin (egg yolk), α-casein (bovine milk), β-casein (bovine milk), ovalbumin (chicken egg), α-lactalbumin (bovine milk) and trypsin inhibitor (soybean).

Adenosine-5′-monophosphate (AMP), adenosine-5′-diphosphate (ADP) and adenosine-5′-triphosphate (ATP) were obtained from Yamasa Shoyu (Chiba, Japan).

DNA (calf thymus) and tRNA (baker's yeast) were obtained from Sigma.

Preparation of pyrophosphates

Analytical-reagent grade chemicals and distilled water were used throughout. As starting materials for spray-pyrolysis, five kinds of slurrys with composition of CaPP, SrPP, MgPP, MnPP and ZrPP were prepared by vigorously mixing solution A with solution B as summarized in Table I. After the reaction, additional methanol

TABLE I

SOLUTIONS USED FOR PREPARATION OF PYROPHOSPHATE SLURRY

Slurry	Solution A	Solution B
$Ca_2P_2O_7$	$Ca(OH)_2$ in water	$H_4P_2O_7$ in methanol
$Sr_2P_2O_7$	$Sr(OH)_2$ in water	$H_4P_2O_7$ in methanol
$Mg_2P_2O_7$	$3MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$ in water	$H_4P_2O_7$ in water
$Mn_2P_2O_7$	$MnCO_3 \cdot nH_2O$ in water	$H_4P_2O_7$ in water
ZrP_2O_7	$ZrO(NO_3)_2 + HNO_3$ in water	H_3PO_4 in methanol

or methanol–water mixed solution was added in order to adjust the slurry to a suitable concentration for spraying and to a desirable pyrolysis temperature by the combustion of methanol. The PPi slurry thus obtained was spray-pyrolysed by the method developed in our laboratory for HAP preparation [11]. The spherical powders produced were then heat treated at 800°C for 4 h in air. Only particles of 4–10 μm classified using an air classifier were used as packing materials.

Preparation of comparative hydroxyapatite sample

In order to compare PPi chromatography with HAP chromatography, a spherical HAP packing material was prepared by spray-pyrolysing a slurry with an HAP composition, classifying the powdery product (4–10 μm), and heat treating it at 800°C in air [11,12].

Analysis of products

The crystalline phases of both the spray-pyrolysed powders and the heat-treated powders were identified by X-ray diffraction analysis using Cu Kα radiation with a RAD III instrument (Rigaku, Tokyo, Japan). The crystallinity was evaluated from the broadening of X-ray line profiles. The shape and size of the powders and products were observed with an Alpha 25 scanning electron microscope (SEM) (Akashi, Tokyo, Japan). The specific surface area of the products was measured by the BET method from measurement of the adsorption of nitrogen at liquid nitrogen temperature. The pore size distribution of the products was determined with a Pore Sizer 9310 mercury porosimeter (Micromeritics, Norcross, GA, USA).

Chromatographic procedure

The spherical PPi packing materials and comparative HAP sample were packed in a stainless-steel tube (100 mm × 8 mm I.D.) under a 100–300 kg/cm^2 pressure using the slurry packing method.

All chromatographic measurements were carried out at room temperature with a system consisting of a model L-5000 LC controller, a Model 655A-11 HPLC pump, a Model 655A-21

variable-wavelength UV detector and a Model D-2500 integrator (Hitachi, Tokyo, Japan).

Samples of proteins, nucleotides and nucleic acids dissolved in 0.001 M sodium phosphate buffer (pH 6.8) were applied to the PPi columns. The bound samples were then eluted with linear gradients starting from 0.001 M sodium phosphate buffer (pH 6.8) at a flow-rate of 1 ml/min. The gradients were $4.83 \cdot 10^{-3}$ M/min for protein elution and $3.25 \cdot 10^{-3}$ M/min for nucleotide and nucleic acid elution. The sample elution was monitored at 280 nm for proteins and at 260 nm for nucleotides and nucleic acids. The column was re-equilibrated by washing for 30 min with the initial buffer.

RESULTS AND DISCUSSION

Properties of pyrophosphates obtained using the spray-pyrolysis method

The droplets produced by spraying a PPi slurry were instantaneously dried and sintered into a spherical and porous material by the heat of combustion of the vaporized methanol. All the spray-pyrolysed materials contained two phases of amorphous and crystalline states. By heat treating these spray-pyrolysed materials at 800°C for 4 h, the completely crystallized products were
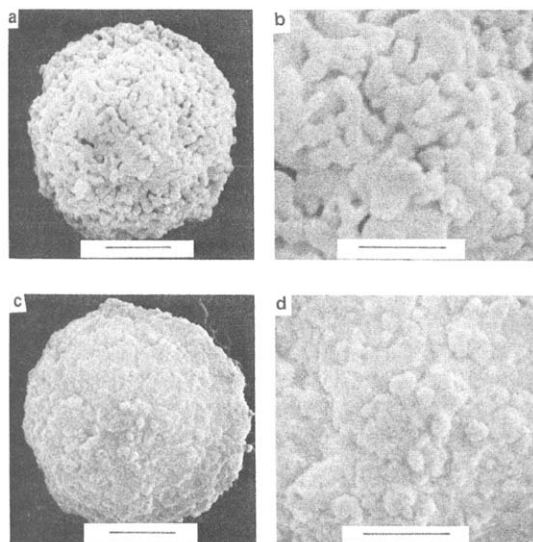

Fig. 1. Scanning electron microphotographs of the appearance and surface texture of the products of (a and b) β-MgPP and (c and d) ZrPP. The bar indicates 2 μm in (a) and (c) and 1 μm in (b) and (d).

obtained as β-Mg$_2$P$_2$O$_7$ (β-MgPP), β-Ca$_2$P$_2$O$_7$ (β-CaPP), α-Sr$_2$P$_2$O$_7$ (α-SrPP), Mn$_2$P$_2$O$_7$ (MnPP) and ZrP$_2$O$_7$ (ZrPP).

Fig. 1a and c show the appearance of the β-MgPP and ZrPP products. The surface texture of ZrPP (Fig. 1d) consisted of a denser aggregate of fine (0.1–0.4 μm) primary particles than that of the β-MgPP product (Fig. 1b). The other three PPi products showed an appearance similar to the β-MgPP product.

The data on specific surface area and pore size distribution are summarized in Table II. The ZrPP product had a larger specific surface area and a smaller pore size than those of the other PPi products. The other four PPi products were not very different from each other in these properties.

Chromatography of proteins

Elution behaviour. Table III shows the elution molarities of the tested proteins with isoelectric points (pI) between 4.3 and 11.4, obtained by using the five kinds of PPi columns. The comparative data on the HAP column, obtained under the same chromatographic conditions are given in the last column.

The basic proteins with p$I > 7$ were retained by and eluted from all the PPi columns. The chromatographic properties of the β-MgPP, β-CaPP and α-SrPP columns were similar to each other and to those of the HAP column. Within the concentration range 0.02–0.19 M, the elution order of the basic proteins was almost the same between the three columns. The properties of the MnPP column were also similar to those of

TABLE II

PROPERTIES OF HEAT-TREATED PYROPHOSPHATE PRODUCTS

Product	Crystal system	Specific surface area (m^2/g)	Pore size distribution (Å)
β-Mg$_2$P$_2$O$_7$	Monoclinic	1.3	1500–4000
β-Ca$_2$P$_2$O$_7$	Tetragonal	4.6	500–2000
α-Sr$_2$P$_2$O$_7$	Orthorhombic	1.8	400–2000
Mn$_2$P$_2$O$_7$	Monoclinic	2.3	1000–3000
ZrP$_2$O$_7$	Cubic	44.3	50–200

TABLE III

ELUTION OF PROTEINS FROM PYROPHOSPHATES AND HYDROXYAPATITE

For details of measurements, see Experimental.

Protein	pI	Elution molarity (M)					
		β-MgPP (monoclinic)	β-CaPP (tetragonal)	α-SrPP (orthorhombic)	MnPP (monoclinic)	ZrPP (cubic)	HAP (hexagonal)
Trypsin inhibitor	4.3–4.6	N.r.[a]	0.04	0.02	0.02	N.r.	0.04
α-Lactalbumin	4.5	N.r.	0.03	0.02	0.03	N.r.	0.04
Ovalbumin	4.6	N.r.	0.02	0.01	0.02	N.r.	0.02
β-Casein		N.r.	0.18	0.24	>0.8	0.11	0.11
α-Casein		N.r.	0.32	0.30	>0.8	>0.8	0.20
Phosvitin	4.8	N.r.	>0.8	>0.8	>0.8	N.r.	>0.8
Albumin	4.7–4.9	N.r.	0.05	0.04	0.04	N.r.	0.06
β-Lactoglobulin A	5.1	0.15	0.12	0.12	0.24	0.23	0.19
β-Lactoglobulin B	5.2	0.18	0.13	0.14	0.25	0.26	0.20
Holo-transferrin (human)	5.2	N.r.	0.02	0.02	0.06	0.09	0.06
Apo-transferrin (human)	5.5	N.r.	0.02	0.01	0.06	0.09	0.06
Catalase	5.5	0.02	0.06	0.07	0.09	N.r.	0.09
Conalbumin (iron complex)	6.0–6.8	0.08	0.10	0.06	0.11	0.12	0.12
Conalbumin (iron poor)	6.0–6.8	0.06	0.08	0.04	0.11	0.12	0.11
Haemoglobin	6.8–7.0	0.06	0.08	0.05	0.11	0.11	0.09
Myoglobin	8.1–8.2	0.02	0.04	0.02	0.06	0.08	0.08
α-Chymotrypsin	8.1–8.6	0.08	0.11	0.08	0.13	0.22	0.13
Papain	8.8–9.5	0.05	0.07	0.05	0.07	0.10	0.08
α-Chymotrypsinogen A	9.5	0.09	0.12	0.09	0.13	0.21	0.13
Ribonuclease A	9.5–9.6	0.08	0.09	0.06	0.13	0.27	0.11
Cytochrome c (reduced)	10.1	0.15	0.17	0.13		0.43	0.19
Cytochrome c (oxidized)		0.17	0.19	0.14	0.23	0.46	0.21
Lysozyme	11.0–11.4	0.08	0.12	0.08	0.12	0.19	0.12

[a] N.r. indicates no retention under the experimental conditions.

the above three PPi columns. However, this column was chemically unstable for repeated use in HPLC. The ZrPP column had a high binding ability for basic proteins, and molarities about twice those of the other PPi columns were required for the elution of proteins with pI > 9.5. For the elution of cytochrome c, two peaks due to reduced and oxidized components were resolved by the PPi columns examined, except for the MnPP column.

Acidic proteins, on the other hand, showed various retention behaviours depending on the kind of PPi. The β-CaPP, α-SrPP and MnPP columns could retain most of the tested acidic proteins with pI < 7, and their chromatography was similar to that of HAP. The chromatograms of acidic proteins obtained using the MnPP

column, however, showed an increase in the baseline with a gradient of phosphate concentration, which is probably due to the unstable chemical properties of this column. In contrast, the β-MgPP and ZrPP columns could not retain the acidic proteins with pI < 6 except for a few proteins: the β-MgPP column retained only β-lactoglobulin A and β-lactoglobulin B, whereas the ZrPP column retained transferrins and β-lactoglobulins A and B. β-Lactoglobulins A and B, in spite of their acidic proteins, required elution molarities higher than those for most basic proteins during all PPi chromatography. It is interesting that the β-MgPP and ZrPP columns could not retain phosphoproteins which interact strongly with the HAP matrix because of the presence of the phosphate groups on the mole-

cule [6]. With these properties, one can expect the β-MgPP and ZrPP columns to be applied to the group separation of basic proteins from most acidic proteins.

As Table III reveals, the correlations between the elution molarity and pI among the basic proteins are weak in all instances. The same kind of weak correlations among the acidic proteins can be obtained, except for β-lactoglobulins A and B, with regard to β-CaPP, α-SrPP and MnPP chromatography. Hence it is suggested that PPi chromatography is not very sensitive to the total charge per protein molecule. On the other hand, it appears that the PPi crystals, in addition to HAP, can discriminate among the different structures existing in protein molecules with very similar dimensions, shapes, isoelectric points, etc., although the crystal structure of PPi is different from that of HAP. For example, all the PPi columns separated β-lactoglobulins A and B, which are known to have a slight difference in the primary structure of each protein.

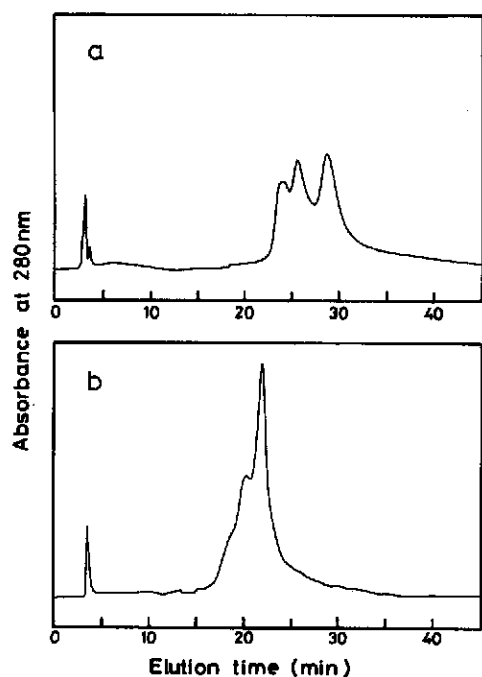Figs. 2 and 3 illustrate chromatograms of

Fig. 3. Chromatograms of conalbumin (iron poor) obtained using (a) ZrPP and (b) β-CaPP columns. Elution conditions as in Fig. 2.

proteins with pI values near 7. For bovine haemoglobin, the ZrPP column gave a good resolution of three peaks (Fig. 2a). The chromatographic resolution obtained using the other PPi columns was inferior to this; Fig. 2b, obtained using the β-CaPP column, is a typical example. For the elution of conalbumin (iron-poor), only one peak was obtained using the ZrPP column (Fig. 3a), whereas two peaks were resolved by the β-CaPP column (Fig. 3b).

Fig. 4 illustrates chromatograms of γ-globulin (bovine) obtained using the PPi columns and a comparative HAP column. It was found that each column gave a characteristic elution profile and different chromatographic resolution among the different components of γ-globulin. For example, the chromatographic resolution obtained using the β-CaPP column (Fig. 4b) is superior to that obtained using the α-SrPP and HAP columns (Fig. 4c and f). During ZrPP chromatography, a different elution profile was obtained consisting of two major sharp peaks (Fig. 4e).

The β-CaPP and α-SrPP columns gave different chromatograms for transferrin (bovine; holo

Fig. 2. Chromatograms of haemoglobin obtained using (a) ZrPP and (b) β-CaPP columns. Elution conditions: gradient, $4.83 \cdot 10^{-3}$ M/min from 0.001 to 0.3 M sodium phosphate buffer (pH 6.8); flow-rate, 1 ml/min.
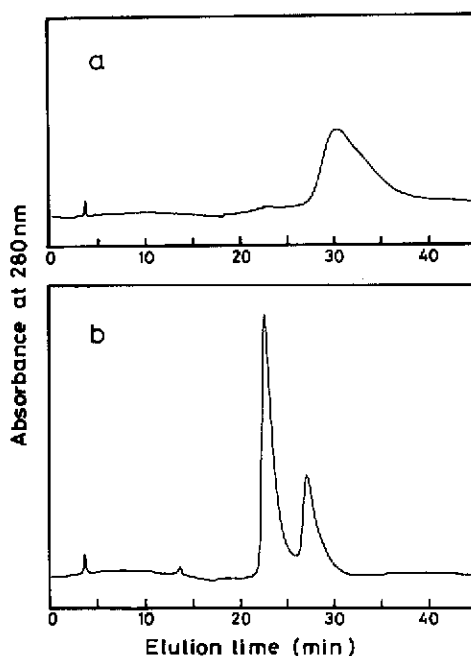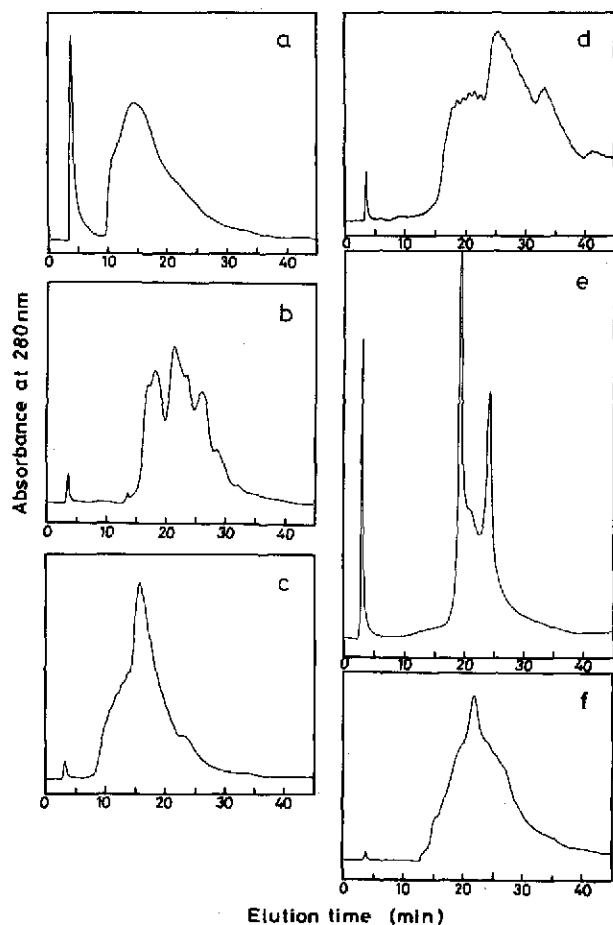
Fig. 4. Chromatograms of γ-globulin (bovine) obtained using (a) β-MgPP, (b) β-CaPP, (c) α-SrPP, (d) MnPP, (e) ZrPP and (f) HAP columns. Elution conditions as in Fig. 2.

Fig. 5. Chromatograms of transferrin (bovine; holo type) obtained using (a) β-CaPP and (b) α-SrPP columns. Elution conditions as in Fig. 2.

type). The chromatogram of transferrin using the β-CaPP column consisted of two major peaks with sharp profiles and some smaller peaks on both sides (Fig. 5a). In contrast, the chromatogram of transferrin using the α-SrPP column showed a broad peak profile overall, which consisted of many different components eluted within the concentration range 0.02–0.12 M (Fig. 5b).

Application to separation of γ-globulin from other serum proteins. A mixture of γ-globulin (human), albumin (human serum), transferrin (human; apo type) and transferrin (human; holo type) was applied to the β-MgPP column. The elution was carried out as described in Fig. 6.
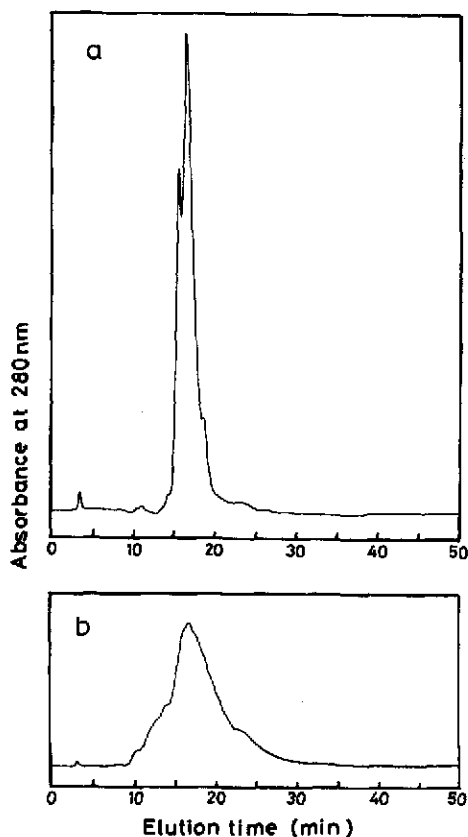
Samples from the peaks thus obtained were identified based on their typical elution molarity and profile by running the single compounds separately. As was expected, the chromatographic separation of γ-globulin from the other serum proteins was achieved (Fig. 6). Albumin and two transferrins were completely eluted with the initial buffer (peak 1), whereas γ-globulin was eluted in the concentration range 0.02–0.19 M (peak 2).

Chromatography of nucleotides and nucleic acids

As Table IV reveals, PPi chromatography of nucleotides (*i.e.*, AMP, ADP and ATP) and nucleic acids (*i.e.*, DNA and tRNA) depended on the kind of PPi column. For example, the retention behaviours of nucleotides and nucleic acids were similar on the β-CaPP, α-SrPP and
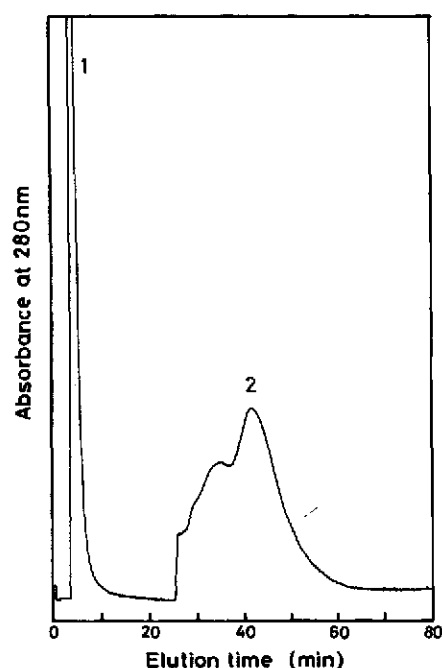
Fig. 6. Separation of a protein mixture containing albumin (human serum), transferrin (human; apo type), transferrin (human; holo type) and γ-globulin (human) using the β-MgPP column. The concentration of sodium phosphate (pH 6.8) was maintained at 0.001 M for 15 min, then a 60-min linear gradient from 0.001 to 0.3 M was started. Peak 1 is due to albumin and two transferrins and peak 2 is due to γ-globulin.

HAP columns. The β-MgPP column could not retain nucleotides but retained nucleic acids, whereas the ZrPP column retained nucleotides but could not retain nucleic acids. However, all chromatograms of the nucleotides obtained using the ZrPP column consisted of a similar profile with two broad peaks and with almost the same retention times between the three nucleotides. Hence it is suggested that the ZrPP crystal cannot discriminate between nucleotides based on the difference in the number of phosphate groups.

DNA retention on the β-MgPP and MnPP columns was stronger than that on the other PPi and HAP columns.

Fig. 7 illustrates the chromatogram of tRNA obtained using the β-CaPP column. Within the main broad peak eluted at about 0.1–0.3 M, good chromatographic resolution was obtained among different components.

From these results, it can be suggested that the difference in crystal structure and component metal ion among the PPi crystals affects the adsorption of nucleotides and nucleic acids: adsorption takes place when the conditions of positively charged adsorbing sites, characterized not only by the geometric arrangement of the metal ion but also by the electrochemical property of the metal ion, are suitable for the adsorp-

TABLE IV

ELUTION OF NUCLEOTIDES AND NUCLEIC ACIDS FROM PYROPHOSPHATES AND HYDROXYAPATITE

For details of measurements, see Experimental.

Sample	Elution molarity (M)					
	β-MgPP (monoclinic)	β-CaPP (tetragonal)	α-SrPP (orthorhombic)	MnPP (monoclinic)	ZrPP (cubic)	HAP (hexagonal)
AMP	N.r.[a]	N.r.	N.r.	N.r.	<0.01	N.r.
ADP	N.r.	<0.01	0.02	0.02	<0.01	0.3
ATP	N.r.	0.05	0.09	0.02	<0.01	0.08
tRNA	0.03–0.11[b]	0.11–0.31	0.04–0.14	0.01–0.09	N.r.	0.01–0.15
DNA	>0.8	0.13–0.18	0.16–0.24	>0.8	N.r.	0.21–0.29

[a] N.r. indicates no retention under the experimental conditions.
[b] For nucleic acids with a broad chromatographic profile, the molarity range, not the molarity at the highest peak, is indicated.
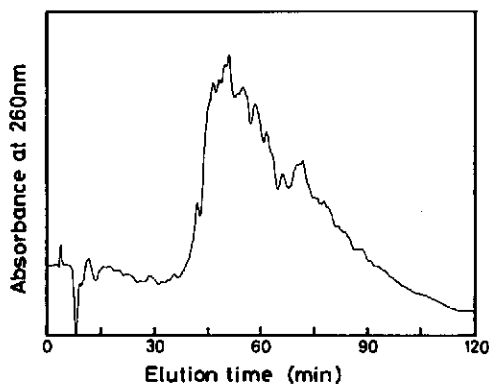
Fig. 7. Chromatogram of tRNA obtained using the β-CaPP column. Elution conditions: gradient, $3.25 \cdot 10^{-3}$ M/min from 0.001 to 0.4 M sodium phosphate buffer (pH 6.8); flow-rate, 1 ml/min.

tion of a molecule with negative phosphate groups.

Influence of surface structure of the PPi crystal

PPi is an inorganic crystal composed of a definite lattice of metal ions and pyrophosphate groups, and its surface presents a complex mosaic of charge depending on the structure of the PPi crystal. Hence the adsorption of molecules such as proteins should depend on the structure of the PPi crystal and the kind of metal ion.

Ca and Sr ions are metal components that can form pure single-cation HAP [*i.e.*, $Ca_{10}(PO_4)_6(OH)_2$ and $Sr_{10}(PO_4)_6(OH)_2$, respectively]. Hence the possibility cannot be excluded that β-CaPP and α-SrPP crystals may be hydrolysed to orthophosphates in a phosphate buffer, followed by further precipitate formation of HAP on their PPi crystal surfaces. If that is the case, then their chromatography should be similar to that on HAP. In fact, the β-CaPP and α-SrPP columns did show elution behaviours similar to that in HAP chromatography with regard not only to basic proteins but also to acidic proteins, nucleotides and nucleic acids.

In contrast, Mg, Mn and Zr ions cannot form pure single-cation HAPs and can only be partially substituted in a parent HAP crystal [13]. Thus, the surfaces of β-MgPP, MnPP and ZrPP would maintain their intrinsic crystal structures under the conditions of this experiment. Their chro-

matographic behaviours should differ from that of HAP, and they should also differ between these three columns. In fact, various elution behaviours of acidic proteins, nucleotides and nucleic acids were obtained, depending on the kind of PPi column, as described in the previous sections. However, with regard to basic proteins, similar chromatographic behaviour between the three columns was obtained, which was also similar to that of HAP.

Moreover, it is interesting that the above-mentioned chromatographic properties of ZrPP are similar to those of zirconium oxide under the condition of the presence of a strong Lewis base such as fluoride or phosphate in the mobile phase [14,15].

A reasonable explanation has not yet been obtained for the different chromatographic effects between PPi crystals with different structures and also between PPi crystals and HAP crystal. It cannot be concluded that PPi crystal surfaces change to the HAP crystal structure by hydrolysis in a buffer merely because the elution behaviour of basic proteins during all PPi chromatography is similar to that of HAP. A possible interpretation of these experimental results might be as follows. The basic proteins are adsorbed by electrostatic interaction. Hence, even though there is a slight discrepancy in geometric arrangement between adsorption groups on the molecule and adsorbing sites on the crystal, a molecule with a flexible structure can be adsorbed on the crystal by slightly modifying the molecular shape. This implies that the PPi crystal does not necessarily need to have the same geometric arrangement of adsorbing sites as the HAP structure. Further, as far as HAP and PPi crystals are concerned, the negatively charged adsorbing sites are always formed by oxygen atoms belonging to the phosphate or pyrophosphate ions. This seems to explain the similar values of the elution molarity for the same basic proteins between the PPi columns and also between the HAP and PPi columns. In contrast, the acidic proteins are considered to be adsorbed in a specific manner by complexation of their adsorption groups to adsorbing sites that are formed by metal ions on the crystal, so that the adsorption is more strongly influenced by the

difference in the geometric arrangement between adsorbing sites and adsorption groups. Moreover, it is likely that the difference in metal ions among the PPi crystals causes the different elution behaviour for the same acidic protein.

In conclusion, it can be expected from the experimental results that these pyrophosphates will provide useful packing materials for specific applications such as the single-step separation of γ-globulin from other serum proteins, which can be achieved using the β-MgPP column. Moreover, β-MgPP and ZrPP crystals seem to be suitable materials for the group separation of basic proteins from acidic proteins or nucleic acids. Except for the MnPP column, the PPi columns prepared by the spray-pyrolysis method in this study were mechanically strong enough to stand as many as 100 chromatographic cycles.

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