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Chromatographic characterization of a phosphate-modified zirconia support for bio-chromatographic applications

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ABSTRACT

A phosphate-modified zirconia was investigated for its potential use as a high-performance inorganic cation-exchange support for the separation of proteins. This phosphate modification effectively blocks the sites responsible for the strong interactions of certain Lewis bases with the zirconia surface. It provides a more "bio-compatible" stationary phase, resulting in high recoveries for proteins and enzymes and retention of their enzymatic activity. The stability, loading capacity, selectivity, efficiency and separation mechanism on the phosphate-modified zirconia are reported. These studies have shown that phosphate-modified zirconia is a useful high-performance ion-exchange support for the separation of cationic proteins and for blocking the sites responsible for the high affinity of zirconia towards certain anions. This makes the phosphate modification interesting in its own right and as an intermediate stage for the development of other zirconia-based chromatographic supports.

INTRODUCTION

As stated in the preceding paper [1], protein separations on porous zirconia have recently become feasible with the development of particles that are mechanically stable and have sufficiently wide pores to avoid steric exclusion of large biomolecules. Obviously, if the separation of small solutes containing a single carboxylate group requires the presence of inorganic phosphate in the mobile phase to block the strong interactions with the surface, the separation of large biopolymers containing many such groups will be extremely problematic. Indeed, in prior work [2], elution of myoglobin and bovine serum albumin from zirconia required a pH 10 eluent and the only partial separation was achieved. Very poor efficiency and peak shapes were observed for proteins despite the presence of phosphate in the mobile phase.

The aim of this work was to modify chemically the surface of zirconium oxide spherules in order to remove these undesirable interactions without seriously compromising the mechanical and chemical stability of the particle. The strong affinity of zirconia for phosphate [3] suggested that a phosphate modification of the surface should be a reasonable approach. A phosphate modification of the zirconia particles that effectively blocks the strong sites responsible for the Lewis base interactions might provide a more "bio-compatible" stationary phase, suitable for the separation of proteins and other biologically important molecules. The widespread use of the calcium phosphate hydroxyapatite in protein chromatography and zirconium phosphate for transition metal ion separations also suggested that a phosphate-modified zirconia should be of interest as a chromatographic support.

The poor mechanical stability of amorphous and crystalline zirconium phosphates has inhibited their use in high-performance liquid chromatography

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(HPLC). Applications with low-pressure techniques such as paper chromatography and gas chromatography have been reported. There are numerous examples of the separation of transition metals on zirconium phosphates but they are beyond the scope of this work and the reader is referred to reviews on this subject [3–6].

Although a search of the literature revealed no studies on protein adsorption or separations on zirconium phosphates, the adsorption of amino acids on zirconium phosphates has been studied [7–9]. The mechanism of adsorption is dependent on the specific amino acid: basic amino acids such as histidine, lysine and arginine are intercalated into the interlayer region, whereas asparagine and alanine are adsorbed on the surface and not in the interlayer region of the zirconium phosphate. For those amino acids which are intercalated within the zirconium phosphate lattice, there is a corresponding loss of phosphate from the interlayer region of the zirconium phosphate which introduces defects into the lattice. This eventually leads to the collapse of the matrix at higher levels of intercalation. Significantly more lysine is adsorbed per gram of α-zirconium phosphate (1.8 mmol/g) than per gram of γ-zirconium phosphate (1.0 mmol/g). Differences in the interlayer spacing, amount of phosphate lost and maximum uptake of the amino acid between the α- and γ-zirconium phosphates show that the exact structure of the zirconium phosphate and the specific amino acid are important factors in the adsorption process. Owing to the higher surface area of the less crystalline zirconium phosphates, they have a higher capacity for the surface-adsorbed amino acids.

Paper impregnated with zirconium phosphate has been used in the separation of amino acids and alkaloids. Coussio *et al.* [10] studied the separation of several alkaloids on paper saturated with zirconyl oxychloride followed by a phosphoric acid treatment. By varying the concentration of zirconyl oxychloride used to saturate the paper, papers with differing capacities were produced. As most alkaloids do not adsorb appreciably on cellulose, adsorption was attributed to the impregnated zirconium phosphate. Catelli [11] investigated the separation of twelve amino acids on this phase but it lacked the selectivity to resolve them.

Zirconium phosphate has been investigated as a gas chromatographic support where the mechanical stability of the stationary phase is not a critical factor. A ligand-exchange separation of lower aliphatic amines using zirconium phosphate treated with copper(II) chloride was developed by Fujimura and Ando [12]. Allulli *et al.* [13] investigated microcrystalline $Zr(KPO_4)_2$ as a stationary phase in the separation of *n*-alkanes and some chlorohydrocarbons and thiols. Other classes of compounds gave asymmetric peaks and poor quantitative results.

Reversed-phase liquid chromatographic supports have been developed based on $Zr(O_3POR)_2$ where the alkyl group R is butyl, lauryl, octylphenyl or octadecyl [14,15]. As expected, these supports lack the mechanical stability necessary for high-performance supports. Rigney [2] investigated several methods of producing a reversed-phase zirconia-based support including the adsorption of organophosphates on porous zirconia spherules. However, these phases were not chemically stable in aqueous media.

Zirconium phosphate has been the most widely studied of the insoluble acid salts of tetravalent metals owing to its high ion-exchange capacity, although thorium, titanium and cerium phosphates [5] have also been investigated.

Calcium hydroxyapatite, $Ca_2(OH)_2(PO_4)_6$, has been extensively studied for the separation of proteins and other biologically important molecules such as nucleic acids [16–18]. The usual principles of ion exchange do not apply to hydroxyapatite chromatography [19–21] and indeed its popularity is probably due to its unique retention properties. The retention mechanism is complicated and several factors affect retention in hydroxyapatite chromatography, including overall and local charges on the protein, the composition of the loading and eluting buffers and the presence of sorbed counter ions on the support. General non-specific electrostatic interactions take place between the amino groups of proteins and the negative phosphate ions on the hydroxylapatite. The carboxyl groups of proteins may specifically complex with calcium sites provided that there are areas of significant carboxyl group density on the protein. The individual interactions are weak enough that many such interactions are required for retention. Small molecules are thus not generally retained on hydroxyapatite columns. There is, however, no correlation between the molecular weight and the retention of larger bio-

molecules on hydroxyapatite. Denatured proteins are generally much less retained than proteins in their native state [19].

A significant advantage of hydroxyapatite supports is their "bio-compatibility". Hydroxyapatite is a major component of bones and teeth, and synthetic hydroxyapatite have been used in prosthetics [22]. Typically a phosphate gradient at neutral pH is used to elute the proteins. Such gradients are much less likely to denature proteins than the conditions typically used in reversed-phase chromatography.

The usefulness of traditional hydroxyapatite chromatography is limited by practical considerations despite its unique retention characteristics. Retention characteristics on hydroxyapatite are extremely preparation dependent [23,24] and producing several batches of hydroxyapatite with the same retention characteristics has been problematic. The mechanical stability of conventional hydroxyapatite is poor and hydroxyapatite crystals break down even during gravity flow column operation, causing column bed collapse and a reduction in flow.

Recently, several manufacturers have introduced hydroxyapatite particles with improved mechanical and hydrodynamic properties, making hydroxyapatite HPLC possible [25–27]. These hydroxyapatite supports are limited, however, in several respects. They may only be used within the limited pH range of 5.5–10.5 and at pressures up to 3000 p.s.i. All of these columns require the use of expensive guard columns and the presence of phosphate and calcium ion in the mobile phase to prevent chemical degradation of the analytical column.

EXPERIMENTAL

All chemicals were of analytical-reagent grade or better. Calcium chloride dihydrate [10035-04-8] was obtained from Fisher Scientific (Fairlawn, NJ, USA), 50% sodium hydroxide solution [1310-73-2] from Curtin Matheson Scientific (Houston, TX, USA), concentrated hydrochloric acid [7647-01-0] from EM Science (Gibbstown, NJ, USA) and 3-[N-morpholino]propanesulphonic acid (MOPS) [1132-61-2] from Sigma (St. Louis, MO, USA). Potassium phosphate dibasic [7758-11-4], potassium phosphate monobasic [7778-77-0], potassium chloride [7447-40-7], potassium acetate [127-08-2], sodium phosphate dibasic heptahydrate [7782-85-6], ammonium phosphate dibasic [7783-28-0] and benzoic acid [65-85-0] were obtained from Mallinkrodt (Paris, KY, USA). Myoglobin (equine skeletal muscle), lysozyme (chicken egg white), α-chymotrypsin (bovine pancreas), ribonuclease A (bovine pancreas), bovine serum albumin (BSA), transferrin (human), cytochrome *c* (type VI from horse heart), β-lactoglobulin (bovine milk), insulin (bovine pancreas), alcohol dehydrogenase (equine liver), hemoglobin-A_0 (human), hemoglobin-A_2 (human) and hemoglobin-A_s (human) were obtained from Sigma and were used without further purification. The preparation of the 0.1 *M* phosphoric acid-treated zirconia was described in the preceding paper [1].

Several chromatographic systems were used throughout the course of this work. System I consisted of an IBM Instruments (Danbury, CT, USA) Model 9533 ternary liquid chromatograph with a Rheodyne (Berkeley, CA, USA) Model 7125 injector and an IBM Instruments Model 9522 fixed-wavelength UV absorbance detector (254 or 280 nm). System II consisted of a Perkin-Elmer (Norwalk, CT, USA) Series 3B binary gradient liquid chromatograph with a Rheodyne Model 7010 injector and a Perkin-Elmer LC-15 fixed-wavelength UV absorbance detector (254 or 280 nm). Unless indicated otherwise, an IBM Instruments Series 9000 laboratory computer with the chromatography applications package (version 1.4) was used for data collection with systems I and II. System III consisted of a Hewlett-Packard (Palo Alto, CA, USA) Model 1090L binary gradient liquid chromatograph with the Model 79847A autosampler, Model 79846A variable-volume injector and Model 79881A UV filter absorbance detector. A Hewlett-Packard Model 3393A reporting integrator was used for data collection. System IV consisted of a Hewlett-Packard Model 1090M ternary gradient liquid chromatograph with the Model 79847A autosampler, Model 79846A variable-volume injector and Model 79880A diode-array UV–VIS absorbance detector installed. A Hewlett-Packard Model 9133 ChemStation with HP 79995A chromatography software was used for data collection and control of the liquid chromatograph. For convenience, these systems will be identified by their assigned Roman numeral as described above.

Stainless-steel (316) column blanks (Alltech, Deerfield, IL, USA), 5 cm in length and 0.46 cm I.D.,

were used for high-pressure studies. Stainless-steel frits (2 μm) were used for small solute studies and 2-μm screens were used for protein separations to minimize the loss of protein. The particles were slurried in isopropanol and thoroughly degassed by sonication and application of a vacuum before packing. Particles with nominal pore diameters of 300 Å or greater were packed at 4500 p.s.i. using the stirred upward slurry technique, whereas particles with smaller pore diameters were generally packed at 6000 p.s.i.

During the course of this work, phosphate buffer gradients were routinely used. The blank gradient run contained two large system peaks: one peak at the beginning of the gradient and one peak after the gradient program returned to the initial phosphate concentration. The size of the first peak depends on the initial phosphate concentration of the gradient and disappears at initial phosphate concentrations of about 25 m*M*. Passing the phosphate buffer solutions over a Chelex 100 analytical-reagent grade chelating resin (Bio-Rad Labs., Richmond, CA, USA) before use decreased the background absorbance of the phosphate buffers but did not eliminate the system peaks.

Additional steps were then taken to reduce the amount of any impurities in the salts. These included rinsing all glassware with concentrated hydrochloric acid prior to use, the use of ultrapure sodium phosphate salts (99.999%) (Aldrich, Milwaukee, WI, USA) and the use of alternative phosphate sources such as ultrapure phosphoric acid. As there was no significant improvement in the size of the system peaks, the use of such expensive reagents was not justified and further studies were done using phosphate buffers prepared from analytical-reagent grade potassium salts. They were then passed over a Chelex-100 column. All buffer solutions were filtered for particulate matter using a Millipore (type HA) 0.45-μm membrane filter prior to use. To avoid the appearance of the large system peak at the beginning of the gradient, an initial phosphate concentration of 50 m*M* was used. Tentatively we assume that the isotherm for the adsorption of phosphate on the surface is saturated above about 25–50 m*M* and hence the system peaks disappear if the phosphate concentration is maintained above this level.

In situ column treatments were accomplished using an Altex (Beckman Instruments, San Ramon, CA, USA) Model 110A isocratic HPLC pump to flush the column with the appropriate solution. A Haake (Karlsruhe, Germany) Model FE circulating constant-temperature bath with a glass column jacket was used for elevated temperature studies. The pH of the mobile phase was measured at the detector outlet using a planar pH electrode system designed for this purpose (Lazar Model FTPH1).

The recovery of protein was studied by comparing the amount of protein collected in a control experiment in which the column was replaced with a zero dead volume detector to that obtained with the column in place. The samples were diluted to the same final volume and analyzed by the BCA assay for total protein (BCA and BCA Protein Assay Reagent, instructions 23230, 23225; Pierce, Rockford, IL, USA, enhanced method, 1986). The recovery of the activity of lysozyme was determined similarly using a standard enzyme assay (assay obtained from the customer service department of Sigma).

RESULTS AND DISCUSSION

To determine if phosphate-modified zirconia had any selectivity for different proteins, the chromatographic elution properties of myoglobin, lysozyme, α-chymotrypsin, ribonuclease A, cytochrome *c* and BSA were investigated. A typical chromatogram is shown in Fig. 1. Peak widths and shapes are generally acceptable although some distorted peaks are noted, especially for α-chymotrypsin and cytochrome *c*. Further improvements in particle size distribution, modification and column packing will hopefully improve the efficiency of the support. The broad α-chymotrypsin peak may be the result of self-digestion and the shoulder associated with cytochrome *c* peak is probably due to the presence of the reduced form. The first peak is a system pressure peak and is not completely resolved from the myoglobin peak. A 30-min linear gradient from 50 to 500 m*M* potassium phosphate (dibasic) adjusted to pH 7.0 with hydrochloric acid was used to effect the separation. This is a typical gradient used in hydroxyapatite chromatography of proteins [28]. Significantly broader peaks resulted when potassium acetate was substituted for potassium phosphate.

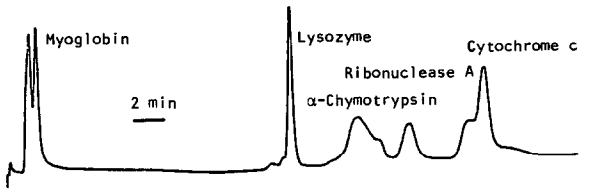

Fig. 1. Chromatographic separation of cationic proteins on phosphate-modified zirconia. Column: 6–11-μm, 300 Å phosphate-modified zirconia (5 × 0.46 cm I.D.). Mobile phase: 30-min linear gradient from 50 to 500 m*M* potassium phosphate dibasic (pH 7.0), 0.5 ml/min. System I (254 nm).

TABLE I

PHYSICAL PROPERTIES OF SELECTED PROTEINS

Protein	Mol. wt.	p*I*
Bovine serum albumin [29]	68 000	4.7
β-Lactoglobulin [30]	35 000	5.5
Insulin [30]	11 466	5.7
Alcohol dehydrogenase [30]	245 000	5.1
Myoglobin [30]	17 500	6.8–7.5
Ribonuclease-A [31]	14 700	9.3
α-Chymotrypsin [29]	25 000	9.8
Cytochrome *c* [29]	11 000	10.2
Lysozyme [29]	14 000	11.0

Retention mechanism

In order to characterize better the mechanism of retention of proteins on the phosphate-modified zirconia, isocratic retention data for several proteins as function of the potassium phosphate buffer concentration were obtained and are shown in Fig. 2. The molecular weights and isoelectric points of the proteins used in this study are given in Table I. The trends in retention suggest that retention is primarily due to cation exchange. At pH > 6, proteins with low p*I* values (*e.g.*, BSA, β-lactoglobulin, alcohol dehydrogenase and insulin) are not retained. In contrast, proteins with higher p*I* values are retained. Retention of retained proteins decrease with increasing competing ion concentration ($[K^+]$), which is also consistent with ion-exchange behavior.

Note that retention does not increase monotonically with the p*I* of the protein. This is a well known phenomena in ion-exchange chromatography [32] and shows that a "net point charge" model is not sufficient to describe the ion-exchange behavior of proteins. The effects of non-homogeneous charge distributions on proteins and specific protein interactions such as agglomeration and conformational changes must be considered [32–34].

To confirm that the retention of the proteins is a function of the potassium ion concentration of the mobile phase and not the phosphate ion concentration, a study similar to that shown in Fig. 2 was done. In this study a constant amount of phosphate

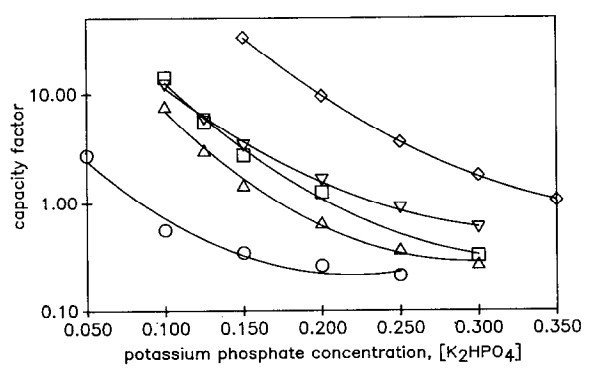

Fig. 2. Isocratic retention data for selected proteins on phosphate-modified zirconia. Column: 20-μm, 300 Å phosphate-modified zirconia (5 × 0.46 cm I.D.). Mobile phase: potassium phosphate dibasic (pH 6.0) at concentration given. System I. ○ = Myoglobin; △ = lysozyme; □ = α-chymotrypsin; ▽ = ribonuclease-A; ◇ = cytochrome *c*.

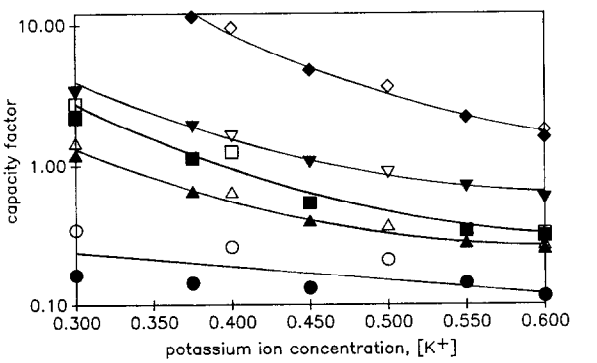

Fig. 3. Isocratic retention data as function of potassium ion concentration. Mobile phase: closed symbols, 0.15 *M* potassium phosphate dibasic (pH 6.0) with additional potassium chloride added to attain the indicated potassium ion concentration, 0.5 ml/min; open symbols, as in Fig. 2. Other parameters and symbols as in Fig. 2.

TABLE II

EFFECT OF VARIOUS CATIONS ON THE ISOCRATIC RETENTION OF SELECTED PROTEINS

Displacing cation	Capacity factor[a]		
	Lysozyme	Ribonuclease-A	α-Chymotrypsin
K^+	0.766 (6.91)	6.27 (6.87)	4.72 (6.87)
Na^+	3.17 (6.89)	10.28 (6.85)	9.99 (6.85)
NH_4^+	0.785 (6.90)	e.n.o.	18.97 (6.90)

[a] Capacity factors determined on 6–11-μm, 300 Å phosphate-modified zirconia, 5 × 0.46 cm I.D. column. Mobile phase: 200 m*M* phosphate buffer with counter ion listed, 0.5 ml/min. System I with HP 3388A recording integrator. Mobile phase pH given in parentheses.

ion (0.15 *M*) as potassium phosphate buffer was present in the mobile phase at all times. The potassium ion concentration of the mobile phase was adjusted by adding known amounts of potassium chloride. The retention data from this study are plotted with closed symbols in Fig. 3. Data from Fig. 2 are replotted with open symbols for comparison. The plot shows that the data from the two studies are in good agreement and the protein retention is independent of the relative amounts of phosphate and chloride, provided that the phosphate level is held above about 0.15 *M*. The scatter in the myoglobin data is attributed to the difficulty in accurately determining small capacity factors. The good agreement in the capacity factors for these two studies shows that the potassium ion is responsible for the elution of the proteins.

The effect of the specific displacing cation on the retention was also examined. Isocratic retention data for three proteins are given in Table II for the potassium, sodium and ammonium displacing cations. The data show that the displacing cation has a significant effect on retention, providing further evidence that the mechanism of retention is cation exchange. Potassium ion has the greatest displacing power of the three, followed by sodium. This trend is consistent with that found for protein chromatography on strong cation-exchange columns [35]. The retention of the proteins may thus be controlled by the choice of the phosphate counter ion and its concentration. More importantly, the potassium ion gives the largest selectivity for all three proteins shown here. As demonstrated by the capacity factors for sodium as the displacing ion, increased retention does not necessarily result in improved resolution. In this case, the potassium ion is obviously the best choice but sodium or ammonium may be better suited for other separations. The presence of phosphate in the mobile phase and the limited solubility of most phosphate salts restrict the number of displacing counter ions that may be used.

The pH of the mobile phase is expected to affect the retention of the proteins by two opposing mechanisms. As the phosphate groups associated with the zirconia surface are most likely responsible for the cation-exchange sites of the phosphate modified zirconia and bound phosphate is a weak ion exchanger, the pH of the mobile phase will determine the effective number of ion-exchange sites. As the pH of the mobile phase is lowered below one of the pK_a values of the surface-bound phosphate, an oxygen of the phosphate becomes protonated and thus reduces the number of possible ion-exchange sites. This, in turn, decreases the retention of proteins on the phase. The pH of the mobile phase also effects the charge on the proteins. The net charge of a protein becomes more positive as the pH of the mobile phase is decreased. In the absence of other effects, this should act to increase the retention of proteins.

To determine which of the above competing

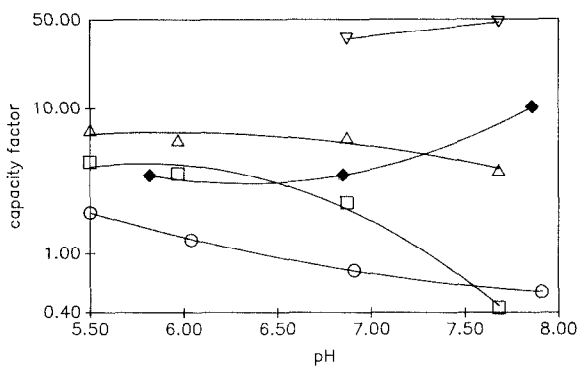

Fig. 4. Effect of mobile phase pH on the retention of selected proteins. Column: 6–11-μm, 300 Å phosphate-modified zirconia (5 × 0.46 cm I.D.). Mobile phase: open symbols, 200 m*M* potassium phosphate dibasic at pH indicated, 0.5 ml/min; closed symbols, 300 m*M* potassium phosphate dibasic at pH given, 0.5 ml/min. System I (254 nm). ○ = Lysozyme; □ = α-chymotrypsin; △ = ribonuclease-A; ▽ and ◆ = cytochrome *c*.

effects dominates the ion-exchange separation of proteins on the phosphate modified zirconia, the capacity factors of four well retained proteins were investigated as a function of mobile phase pH. The retention times of lysozyme, ribonuclease A, α-chymotrypsin and cytochrome *c* were determined isocratically with a 0.2 *M* potassium phosphate buffer solution at nominal pHs of 6.0, 7.0 and 8.0 and are given in Fig. 4.

As expected for complex biopolymers, the exact effect of pH is dependent on the individual protein. Retention increases with decreasing pH for all proteins except cytochrome *c*. We conclude that the protonation of these proteins has a larger effect on retention than does the protonation of the stationary phase. The behavior of cytochrome *c* cannot be explained by this argument. The decrease in retention of cytochrome *c* at the lower pH was confirmed with additional studies with 0.3 *M* potassium phosphate. As the dependence of protein retention on pH varies with the individual proteins, pH will be an important factor in the optimization of protein separations, especially in isocratic separations.

Attempts to separate proteins using a pH gradient were unsuccessful. Anionic proteins such as BSA, alcohol dehydrogenase and insulin were retained at low pH (2) and low phosphate conditions (50 m*M*), but all proteins eluted at the end of the linear gradient and were unresolved. The column thus shows no selectivity between anionic proteins with a pH gradient. BSA did not elute when a 50 m*M* (pH 3.0)–500 m*M* (pH 3.0) linear potassium phosphate gradient was used. This information combined with earlier observations that the mobile phase cation is responsible for the elution of proteins strongly supports the view that the predominant mechanism of retention is cation-exchange.

Phase stability

The stability of the phosphate-modified zirconia is an important consideration in determining its viability as a useful chromatographic support for routine analytical and preparative separations. As discussed above, one must maintain some minimum amount of phosphate in the mobile phase when calcium phosphate (hydroxyapatite) columns are used. Guard columns are recommended and the columns may only be used over a limited pH range.

The possible re-emergence of strong anionic interactions with the surface owing to the desorption of phosphate from the surface was an area of great concern as it would result in poor peak shapes and changing retention times. To determine if some minimum phosphate concentration must be maintained in the mobile phase for these supports, benzoic acid was used as a probe solute. Benzoic acid is not retained on a purely cationic stationary phase. If, however, significant amounts of phosphate were to be lost from the suface, the carboxylic acid group of the benzoic acid will interact with the bare zirconia support resulting in increased retention times and peak widths. By periodically determining the retention time and peak width of benzoic acid on a freshly packed phosphate-modified zirconia column while flushing the column continuously with a phosphate-free mobile phase, the amount of phosphate loss from the surface of the particles can be

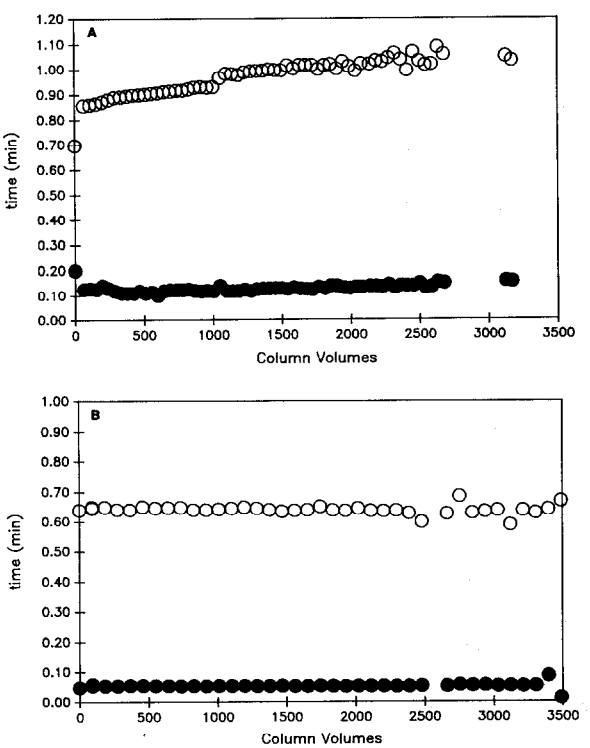

Fig. 5. Stability of the phosphate-modified zirconia using benzoic acid as a probe solute. Column: 10–15-μm, 300 Å phosphate-modified zirconia (5 × 0.46 cm I.D.). Mobile phase: (A) 1 m*M* MOPS buffer (pH 7.0), 0.5 ml/min; (B) 20 m*M* potassium phosphate dibasic (pH 7.0), 0.75 ml/min. System III (254 nm). ○ = Retention time; ● = peak width.

measured. The pH of the mobile phase without phosphate was buffered with 1 m*M* MOPS (pK_a = 7.2).

The retention time and to a lesser extent the peak width of the benzoic acid are affected as the support is exposed to phosphate-free mobile phase (see Fig. 5A). A reasonable explanation for the increase in retention time and peak width is that phosphate is being stripped from the surface exposing the underlying bulk zirconia matrix. Data for the study in which 20 m*M* phosphate was used in the mobile phase are plotted in Fig. 5B. The retention time and peak width of the benzoic acid remained constant throughout the study, indicating that the stationary phase is stable under these conditions. This clearly shows that the presence of 20 m*M* phosphate in the mobile phase is sufficient to maintain a reproducible behavior of the stationary phase.

The stability of the phosphate-modified zirconia in alkaline media was also assessed chromatographically. The retention times of three proteins, lysozyme, α-chymotrypsin and cytochrome *c*, were taken as a measure of the stability of the support to several alkaline treatments. Any significant changes in the retention times of the proteins after base treatment would indicate that the support is not stable under those particular base treatment conditions. Retention data are given in Table III for successively harsher alkaline treatments.

The column appeared to be fairly stable to flushing with 200 m*M* potassium phosphate (pH 11.9) for 1 h, although more extensive treatment would probably result in larger effects on retention as there is already evidence of a slight decrease in retention after 1 h. Previous chemical studies [1] have shown that the support is stable at pH < 10. Increasing the pH of the potassium phosphate solution to 13 had a dramatic effect on protein retention despite the presence of phosphate in the mobile phase. The retention dropped by over 30% for α-chymotrypsin and cytochrome *c*. This is consistent with phosphate desorbing from the surface of the particles as a result

TABLE III

CHROMATOGRAPHIC ALKALINE STABILITY STUDIES OF PHOSPHATE-MODIFIED ZIRCONIA[a]

Treatment conditions	Retention time (min)[a]		
	Lysozyme	α-Chymotrypsin	Cytochrome *c*
200 m*M* K_2HPO_4, pH 7.2, 2 h at 1 ml/min, R.T.[b]	1.223 (0.005)[c]	2.839 (0.07)	17.691 (0.04)
200 m*M* K_2HPO_4, pH 11.9, 1 h at 1 ml/min, R.T.	1.216 (0.003)	2.656 (0.001)	17.455 (0.015)
200 m*M* K_2HPO_4, pH 13.1, 1 h at 1 ml/min, R.T.	1.266 (0.001)	1.732 (0.005)	10.287 (0.01)
0.1 *M* KOH, 1 h at 1 ml/min, R.T.	1.263 (0.002)	1.746 (0.002)	10.015 (0.02)
0.1 *M* KOH, 1 h at 1 ml/min, R.T.	1.220 (0.05)	1.714 (0.004)	9.884 (0.04)
0.1 *M* H_3PO_4–1.0 *M* KCl, 24 h at 0.5 ml/min, R.T.	1.257 (0.002)	1.808 (0.03)	9.872 (0.008)
0.1 *M* H_3PO_4–1.0 *M* KCl, 4 h at 1.0 ml/min, 84°C	1.229 (0.002)	1.923 (0.01)	10.688 (0.03)
0.1 *M* H_3PO_4–1.0 *M* KCl, 12 h at 0.5 ml/min, 94°C	1.205 (0.02)	2.512 (0.01)	15.115 (0.04)

[a] Retention times determined after equilibrating the column with 200 m*M* K_2HPO_4 (pH 7.0) for 2 h at 1 ml/min. Column: 6–11-μm, 300 Å ZrP (0.1), 5 × 0.46 cm I.D. Flow-rate, 1.0 ml/min. System I.

[b] R.T. = Room temperature.

[c] Standard deviations for three injections given in parentheses.

of alkaline attack, resulting in a net decrease in the number of ion-exchange sites and thus a decrease in protein retention. Static stability studies [1] showed that significant amounts of phosphate are released at this pH. However, a comparable degradation in peak shape did not occur. As the peak shapes are not similarly affected, the sites responsible for strong oxyanion interactions appear to lose sorbed phosphate at a much lower rate than other sites on the surface. Additional treatments with potassium hydroxide in the absence of phosphate had little additional effect on protein retention, suggesting that the base-treated support is quasi-stable.

No specific studies to test the mechanical stability of the particles were carried out but it should be noted that during the course of this work (1.5 years) there was no case of bed collapse with routine use. The particles were routinely packed at 4500–6000 p.s.i. without evidence of particle disintegration. Based on these experiences, the mechanical stability of the particles is more than sufficient for high-pressure work.

Column regeneration studies

Attempts to regenerate the support *in situ* are also summarized in Table III. Neither the 24-h flush with neutral 200 m*M* potassium phosphate nor the 4-h flush with 0.1 *M* phosphoric acid–1.0 *M* potassium chloride solution at room temperature restored the original retention properties of the support. Improved retention of the proteins resulted from a 4-h, 84°C flush with 0.1 *M* phosphoric acid solution but the conditions were not sufficient to return the column to its original state. The 12-h, 94°C flush with 0.1 *M* H_3PO_4–1.0 *M* KCl also did not entirely return the column to its original state. The retention of the proteins is greatly increased by the treatment, however, and at least partial *in situ* regeneration of the support should be possible.

This study emphasized the importance of temperature in modifying the surface, as described previously [1]. Flushing the column with a phosphoric acid solution at room temperature had very little effect. Even the 84 and the 94°C treatments fell short of modifying the particles to the extent achieved by refluxing the particles. This is despite the much longer reaction time (12 *vs.* 4 h) for the 94°C case. The additional 10–12°C achieved by refluxing the particles in solutions at a high ionic strength appears to be significant in preparing the phosphate-modified zirconia.

TABLE IV

PROTEIN RECOVERIES ON PHOSPHATE-MODIFIED ZIRCONIA, ZrP (0.1)

Protein	Protein (mg)[a]		Recovery (%)
	Injected solution	Collected fraction	
Ribonuclease-A	0.182	0.173	95 (±5)
Lysozyme	0.207	0.196	95 (±5)
Cytochrome *c*	0.178	0.152	85 (±7)
Lysozyme enzymatic activity			
Total activity of the injected sample		57 (±8)[b]	
Total activity of the collected fraction		62 (±3)	

[a] As determined by the BCA total protein assay. Mean values given for three trials.

[b] Units as defined by the Sigma lysozyme assay.

Recovery studies

A good support must have high recoveries for the solutes it is intended to separate, *i.e.*, very little solute must remain in the column after a separation. Irreversibly bound protein will certainly affect the retention characteristics of the column. In some instances, the protein of interest is present in minute amounts and thus even small absolute amounts of irreversibly bound protein can significantly affect the yield. The protein recoveries are relatively high and could be 100% within the errors of measurement for lysozyme and ribonuclease A (see Table IV). Protein recovery studies should be done with as little protein as possible so that small amounts of adsorbed protein will still be significant in comparison with the amount injected. Although the BCA method significantly increases the detection limit of protein as compared with simply measuring the UV absorbance of the protein solution, it introduces considerable uncertainty in the measurements as it involves many steps.

Lysozyme was used to determine if the phosphate-modified zirconia denatured significant amounts of enzyme. Lysozyme is a "robust" enzyme and hence is not as sensitive a measure of the "bio-compatibility" of the support as we would have liked. However, most enzymes are anionic at neutral pH they are not retained on this support. The enzymatic activity of a

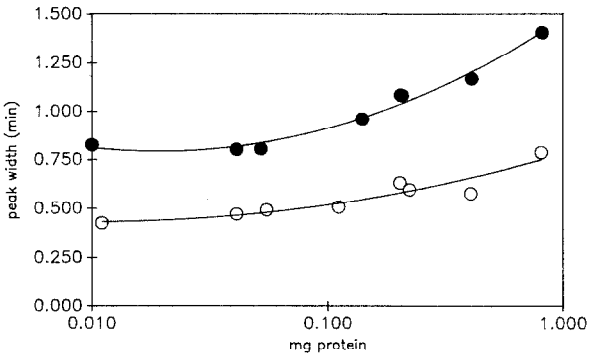

Fig. 6. Loading capacity of the phosphate-modified zirconia. Column: 6–11-μm, 300 Å phosphate-modified zirconia (5 × 0.46 cm I.D.). Mobile phase: 30-min linear gradient from 50 to 500 m*M* potassium phosphate dibasic (pH 7.0), 0.5 ml/min. System I (254 nm) with HP 3388A integrator. ○ = Lysozyme; ● = cytochrome *c*.

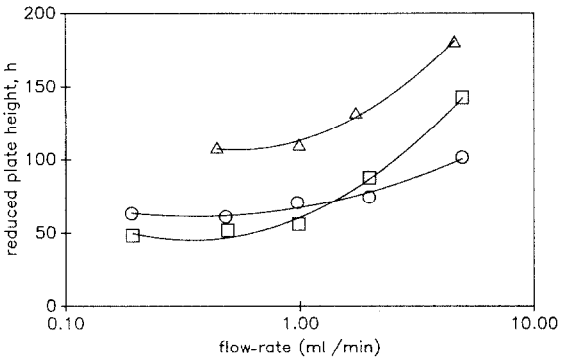

Fig. 7. Reduced plate height, *h*, *vs.* flow-rate for selected proteins on phosphate-modified zirconia. Column: 6–11-μm, 300 Å phosphate-modified zirconia (5 × 0.46 cm I.D.). System II with HP 9133 integrator. (△) Myoglobin, 50 m*M* potassium phosphate dibasic (pH 7.0), k' = 0.64; (○) lysozyme, 150 m*M* potassium phosphate dibasic (pH 7.0), k' = 2.17; (□) cytochrome *c*, 300 m*M* potassium phosphate dibasic (pH 7.0), k' = 6.89. 20-μl injections; 2 mg/ml protein solutions.

lysozyme solution, collected after elution from the support, was compared with an equal amount of the same lysozyme solution that was not exposed to the phosphate-modified zirconia. The data show that adsorption of lysozyme on phosphate-modified zirconia and subsequent desorption do not adversely affect its enzymatic activity. The higher total enzymatic activity of the lysozyme after exposure to the zirconia may be simply explained by the statistical error of the measurements. Alternatively, the phosphate-modified zirconia may have removed some inhibitors from the collected fraction.

Loading capacity and flow-rate studies

The loading capacity of the column was examined and the data are shown in Fig. 6. The plot shows that the column efficiency begins to suffer when more than about 50 μg of protein are injected. As these proteins are well resolved, they were injected as a mixture and thus the loading capacity of the column would be higher if the proteins were injected separately. This loading capacity corresponds to about 60 μg/ml of column volume or 1.49 μg/m^2 (comparisons based on the mass of stationary phase are inappropriate as zirconia is much denser than either silica or polymeric based phases). Loading capacities for comparable carboxymethylsilica-based columns are 30–300 μg/ml of column volume (data for Bio-Sil TSK CM-3-SW [36]). One particular high-performance hydroxyapatite support had a loading capacity of 120 μg/ml column volume [37].

The effect of flow-rate on the efficiency of the column was also examined. Proteins were used in this study as small cationic and anionic solutes are not well retained and proteins will be typically the species of interest. Plate heights were determined isocratically. The potassium phosphate concentration of the mobile phase was adjusted so that a range of capacity factors could be examined. The results are shown in Fig. 7.

The large reduced plate heights shown are not surprising as proteins are being used as probe molecules. Ion-exchange chromatography generally has lower efficiencies than other modes of chromatography [38]. The higher reduced plate heights observed for myoglobin may be due to specific effects with the stationary phase. Based on these

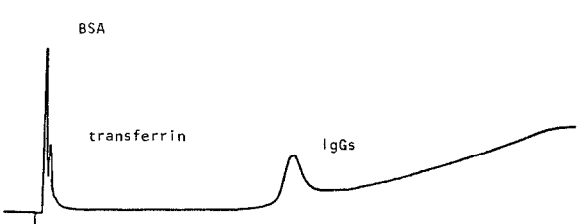

Fig. 8. Separation of IgGs from a modeled fermentation broth. Column: 6–11-μm, 300 Å phosphate-modified zirconia (5 × 0.46 cm I.D.). Mobile phase: 30-min linear gradient from 50 to 500 m*M* sodium phosphate buffer (pH 6.0), 0.5 ml/min. System I.

conditions, the efficiency of the column begins to decrease at an accelerated rate at flow-rates of 1.0 ml/min and higher (linear velocities of 0.12 cm/s and higher).

Applications

All of the separations and chromatographic studies discussed thus far were done in order to understand better the separation mechanism of the proteins on the phosphate-modified zirconia. To demonstrate some practical uses for the support, the preparative-scale separation of murine IgGs from a fermentation broth was examined. The fermentation broth was modeled by a solution of BSA, transferrin and a purified IgG sample generously provided by the Bioprocess Technology Institute at the University of Minnesota. The chromatogram is shown in Fig. 8. As expected, the anionic BSA and transferrin are unretained. The IgGs are retained and can be eluted from the column with the potassium phosphate buffer gradient. Triplicate injections of the same sample solution showed no significant changes in retention times or peak areas. Because only IgG is retained, the loading capacity of the support for this separation is very large.

CONCLUSIONS

These studies on the chromatographic characterization of phosphate-modified zirconia have shown that this substrate has potential as a support for HPLC, particularly for proteins with high p*I* values.

Cation-exchange is the mechanism of separation on this support. This limits the applicability of the support as most proteins and enzymes are anionic at neutral pH. The IgG preparative separation example demonstrates the utility of the support for solutes that are retained. As very few proteins are retained there is little difficulty in resolving the cationic protein of interest from the unwanted components of the sample matrix. Small solutes are likewise easily separated from proteins of interest as they are only retained at very low buffer concentrations.

These studies have shown that phosphate-modified zirconia is a useful high-performance cation-exchange support for the separation of proteins. The phase was found to have sufficient selectivity, efficiency and protein recovery while overcoming the strong oxyanion interactions associated with unmodified zirconia supports.

Phosphate-modified zirconia behaves as a classic cation exchanger and not as a mixed-mode medium analogous to hydroxyapatite, despite spectroscopic evidence of zirconium phosphate formation on the surface. There is no evidence that the support acts in a "hydroxyapatite-like" manner. It appears that interactions between proteins and the zirconium sites on the phosphate-modified zirconia are too strong to be chromatographically useful. Under the mobile phase conditions typically used in this work, it is likely that phosphate ions adsorb on and thus block the zirconium sites on the surface. The high affinity of zirconium phosphate for phosphate ion has been discussed earlier. This explains the cation-exchange-type behavior rather than a hydroxyapatite-like behavior.

The successful blocking of the strong oxyanion interaction sites on the zirconia surface makes the phosphate modification interesting as an intermediate phase in the development of other zirconia-based chromatographic phases. Strong interactions of solutes containing oxyanion functionalities with the bulk zirconia support persist even after zirconia particles have been coated with polybutadiene [2]. It is unlikely that any polymer coating will be dense enough to cover the surface entirely without severely limiting mass transport into the pores. Hence some modification of the zirconia surface will be necessary to block these interactions even when the particles are further modified by polymer coating. The phosphate modification described in this work can serve this purpose.

The susceptibility of the covalent phosphate bonds to hydrolysis at alkaline pH, as evidenced by the solid-state ^{31}P NMR studies [1], is the major drawback of the phosphate-modified zirconia. The chief advantage of the base zirconia support, *i.e.*, its pH stability (>10), is thus lost owing to the instability of the phosphate surface modification. As the phosphate modification is relatively easily done, it is possible at least partially to regenerate the column *in situ* by flushing with hot phosphoric acid solution. When the column becomes fouled, it can be flushed with sodium hydroxide solution to remove any irreversibly bound protein and the phosphate modification. The phosphate modification could then be reapplied by flushing the column with hot

phosphoric acid. The correct conditions for performing an *in situ* phosphate modification of the particles must also be determined.

Several other polybasic acid salts of zirconium have been examined as ion exchangers, including zirconium arsenate, molybdate, tungstate, antimonate, tellurate and silicate [3,4]. Recently, the synthesis of zirconium phosphoborate has been reported [39]. As sugars are known to form borate complexes and this has been successfully exploited in their separation [38], such a phase could be a convenient method for sugar separations.

In conclusion, phosphate-modified zirconia has been shown to be a viable high-performance support for the separation of cationic proteins. In order to take full advantage of the pH stability of the base support, the phosphate modification should be optimized so as to leave the bulk support untouched.

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