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NEW ION-EXCHANGE PACKINGS BASED ON ZIRCONIUM OXIDE SURFACE-STABILIZED, DIOL-BONDED, SILICA SUBSTRATES

R. W. STOUT*, S. I. SIVAKOFF, R. D. RICKER, H. C. PALMER, M. A. JACKSON and T. J. ODIORNE

E. I. du Pont de Nemours & Co., Inc., Biomedical Products Department, Glasgow Site, Building 100, Wilmington, DE 19898 (U.S.A.)

SUMMARY

A new family of silica-based ion-exchange packing materials has been compared in stability and chromatographic performance to similar organic based materials. The new materials are derivatives of the zirconium oxide-stabilized Zorbax® Bio Series GF-250 and GF-450. Long-term stability of the cation-exchange packings has been determined with no detectable loss of the organic surface material. Using a previously published theoretical model, the surface of these materials has been tested with various proteins. Although many results correlate with the model, certain modifications may be necessary if it is to describe all observations. In a practical application of these new packings, monoclonal antibody was purified from ascites fluid and was further analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. These new ion-exchange packing materials function in a manner very similar to packings based on synthetic organic polymers.

INTRODUCTION

Ion-exchange chromatography (IEC) has been in constant use since the introduction of the technique in 1950[1]. The production of synthetic organic resins on an industrial scale has allowed the solution of numerous analytical and preparative problems. Helfferich[2] observed that the number of scientific publications devoted to ion exchange increased exponentially from 1946 to 1955, 1200 papers having appeared by mid-decade. Today, this level is no doubt achieved yearly, if open column separations in the biochemical literature are included.

A forerunner of modern high performance ion-exchange chromatography (HPLC) was initiated by Moore and Stein[3] with the invention of the amino acid analyzer, an event which culminated in the Nobel Prize. The operating pressure required was moderate (*ca.* 100 p.s.i.) by current standards, since the packing particle diameters were large. Nevertheless, the automated device first used in 1951 foreshadowed contemporary instrumentation.

If a particular point in time can be selected as a landmark in advancing chromatographic techniques, the 1967 work of Horváth *et al.*[4] is exemplary. Pellicular

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ion-exchange packings were produced using glass beads on which a thin layer of organic polymer was deposited. The coated beads were subsequently derivatized with ionogenic functional groups. The first application of this revolutionary development was in the separation of nucleotides. Since the organic surface layer was very thin, rapid diffusion of solute molecules lead to short analysis times.

Sample capacity was limited, however, and high operating temperatures were a requirement for acceptable efficiency.

Later, Kirkland[5] developed superficially porous particles under the trademark Zipax®. A crust of spherical silica sol particles was formed on glass beads and was later chemically derivatized. This innovation in packing technology produced highly efficient columns, which allowed rapid analysis of nucleotides and other substances at ambient temperatures.

In principle, pellicular packings are ideal for use in most applications. Spherical shape, controlled porosity, packed-bed geometry, and other factors indicate that this concept achieves nearly optimal conditions. However, materials of this type having mean particle diameters (d_p) of less than 20 μm are not commercially available. In order to decrease analysis time significantly, a reduction of d_p to much less than 10 μm was necessary, as predicted by Knox and Saleem[6]. Due to difficulties with the manufacturing procedures of pellicular particles and lack of materials, smaller porous silica particles, introduced by Majors[7], supplanted the pellicular type. Indeed, when totally porous particles of 3 μm mean diameter or less are employed, the most attractive feature of pellicular particles, namely short diffusion pathway, becomes an intrinsic part of the support, and the use of the impervious core is unnecessary.

Totally porous 10-μm siliceous particles with bonded ion-exchange moieties were introduced by Whatman in the early 70s. Hartwick and Brown[8] applied them extensively to nucleic acid constituents, and Kissinger *et al.*[9] and Stout *et al.*[10] investigated biogenic amines and their metabolites. The separation of oligo-γ-L-glutamates (up to MW *ca.* 1000) was also examined[11]. During the studies on dopamine metabolites and polyglutamates, sample recovery and column stability were examined in detail, and substantial peak tailing and column instability were observed. The lifetime for the Partisil® 10SAX column ranged from two to six weeks[11]. While these microparticulate columns were clearly superior to thin-layer chromatography (TLC) or to pellicular ion-exchange columns in performance, the need for improvement in bonded-phase stability was indicated.

Alpert and Regnier[12] adsorbed polyethyleneimine on porous silica substrates with subsequent cross-linking. This resulted in a material which was more stable in buffered mobile phases than the silica beneath the cross-linked polymer. The criterion for stability was based on changes in column inlet pressure at pH 8.5.

Polymer-based ion-exchange packings have very recently been introduced commercially as Monobeads® by Pharmacia[13]. Column life times were studied by Johansson and Stafström[14] by subjecting the column to 1000 repetitive sample injections. No significant deterioration in column performance was observed. Other ion-exchange packing materials based on organic substrates have been discussed in several articles[15–18].

Numerous approaches are available for the design and synthesis of new ion-exchange packing materials. A relatively large number of critical chromatographic parameters must be balanced so that the final product can perform reasonably well

over a wide range of operating conditions. This paper describes the performance of new ion-exchange packings (Zorbax® Bio Series SCX-300, WCX-300, SAX-300, and WAX-300), based on a hydrophilic siliceous gel filtration support: Zorbax® Bio Series GF-450[19].

EXPERIMENTAL

Chemicals and reagents

Separations of biomolecules were carried out with various buffer systems, containing mixtures of the following: sodium phosphate, potassium phosphate, sodium chloride, (all from Fisher Scientific, Fairlawn, NJ, U.S.A.), sodium acetate (from J. T. Baker, Phillipsburg, NJ, U.S.A.), or Bis-Tris (from Calbiochem-Behring Corporation, La Jolla, CA, U.S.A.).

Buffer pH levels were adjusted to the appropriate value with hydrochloric acid, sodium hydroxide, or potassium hydroxide (all from Fisher). Proteins and test materials for separation were purchased as follows: uridine from Nutritional Biochemicals Corporation (Cleveland, OH, U.S.A.); lysozyme and α-chymotrypsinogen from Calbiochem-Behring; ovalbumin, conalbumin, ribonuclease A, and cytochrome *c* were from Sigma (St. Louis, MO, U.S.A.). Water for all applications was purified by a deionizing system from Continental Water Systems (North Wales, PA, U.S.A.). Samples for chromatography contained 1 mg/ml of each protein in the mixture and approximately 100 μg/ml uridine. Injection volumes were 25 μl.

Instruments

HPLC, requiring inlet pressures greater than 4 MPa, was carried out using a Model 8800 liquid chromatograph (Du Pont, Wilmington, DE, U.S.A.), including a Rheodyne Model 7125 sample injection valve (Rheodyne, Berkeley, CA, U.S.A.). Real-time chromatograms were recorded with a Hewlett-Packard Model 7131A strip-chart recorder. Chromatography at pressures less than 4 MPa was performed with a fast protein liquid chromatography system (Pharmacia, Piscataway, NJ, U.S.A.), including a LCC-500 controller, two P-500 pumps, a mixer, a motor valve MV-7 injector, a UV-1 single-path monitor with a HR-10 flow-cell and 280-nm filter, and a REC-482 recorder. For packing stability studies a third P-500 pump was used in conjunction with the UV-1 detector and REC-482 recorder.

Data system

Analog data were digitized and archived by a Nelson Analytical Series Interface, (Nelson, Cupertino, CA, U.S.A.) and by means of a modified version of Nelson Analytical Chromatography Software on an HP Series 220 Microcomputer (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Columns

The Mono S and Mono Q columns, both HR 5/5, were purchased from Pharmacia. All other columns were slurry packed using proprietary procedures in 8 cm × 6.2 mm I.D. tubes with Zorbax Bio Series GF-250 and GF-450 DIOL-based packings, modified with various ionogenic functional groups by proprietary procedures: Bio Series SCX-150, WCX-150, SAX-150, WAX-150, 6-μm particle diameter,

150-Å pore diameter, specific surface area 144 m^2/g; Bio Series SCX-300, WCX-300, SAX-300, WAX-300, 7.5-μm particle diameter, 300-Å pore diameter, specific surface area 52 m^2/g. For all experiments, pre-columns consisted of either a 2 cm × 2 mm I.D. (Upchurch Scientific, Oak Harbor, WA, U.S.A.) or 4 cm × 4.6 mm I.D. tube, which was packed with 20-μm Zorbax C-8 (Du Pont). The precolumn was installed between the pump and the injection valve to remove any contaminants in the buffers. Elemental analysis of packing materials was performed by Micro-Analysis (Wilmington, DE, U.S.A.).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Sodium dodecyl sulfate chemicals for gel electrophoresis were purchased as a kit, (P/N 161-5200, from Bio-Rad Laboratories, Richmond, CA, U.S.A.). Denaturing SDS-PAGE was performed by the procedures of Laemmli[20].

Ascites fluid was obtained from Colcher *et al.*[21] and used without further treatment.

RESULTS AND DISCUSSION

Stability of silica supports

Amorphous silica is soluble in water[22,23] to the extent of about 100–130 ppm. Since the majority of HPLC packings currently in use are based on this substance, an immediate problem arises with regard to column lifetime. Columns of siliceous packing materials are used most frequently in the reversed-phase mode with hydro-organic mobile phases. High ratios of organic liquids to water significantly reduce the concentration of soluble silicates in chromatographic effluents, thus minimizing the problem in this mode. The silica surface is soluble in alkali, and rapid deterioration is usually observed when pure aqueous buffers at pH >7 are used as mobile phase. This may manifest itself in several ways: (1) soluble silicates can be formed from the surface of the substrate; (2) covalent bonds between surface silicon atoms and ligands can be cleaved; and (3) small clusters of silicates or organosilicates can be dislodged. These processes can produce any or all of the following deleterious results: (1) loss of particle structural integrity resulting in bed collapse; (2) loss of bonded phase, which alters retention (k'), and (3) adsorption due to silanophilic interactions between basic solutes and exposed silanol groups.

Several approaches are currently being utilized to minimize these effects. Organic substrates can be employed which, of course, circumvent the dissolution problem but may introduce hydrophobic interaction phenomena. Gel-filtration columns of synthetic organic polymers are not commonly employed in this chromatographic mode, perhaps for this reason. Agarose-based packings have recently been introduced[24,25], which apparently do not suffer from hydrophobic interactions, but the separation times are quite long (hours) due to the large particle size (*ca.* 30 μm) and lack of sufficient rigidity for high-pressure operation. Bonded-phase silica as a substrate has been predominantly employed for several years in high-performance gel filtration and has been studied in detail[26–33].

One approach to minimizing dissolution of siliceous packing materials is the use of a hydrophilic polymer coating[34]. Another approach has been to cover completely the surface of the silica with a metal oxide, such as zirconia[35], thus improving the water resistance of the glass or silica support.

Since pore volume must be preserved to maximize resolution[36] and thick crusts of a metal oxide or polymer coatings, or both, reduce the value of this critical parameter, a different method of surface deactivation was investigated. Stout and DeStefano[33] observed that silica surfaces could be significantly stabilized against attack from hydroxyl ions by deactivating *geminal* silanol surface groups with zirconium oxide. Very low levels of metal oxide were required (*ca.* 1 μmole/m^2), thus preserving both the pore volume and the stable Si–O–Si bonds used to attach covalently a hydrophilic molecular monolayer. From these studies, it was concluded that the major effect of hydroxyl-ion attack on silica is dissolution and not siloxane–ligand bond cleavage since the zirconium oxide of the treated silica was observed to be stable at pH >9. These packing materials (Du Pont Zorbax Bio Series GF-250, GF-450) were found to be suitable for the gel chromatography of numerous biological macromolecules[19,33], and the inert nature of these substrates immediately suggested their use as ion-exchange supports, after appropriate derivatizations with ionogenic functional groups. The results of this effort form the basis of this paper.

Theoretical considerations

Non-mechanistic chromatographic theory is well established for small molecules[6,37–39]. These models describe band broadening as a function of several geometric properties of the column packing material, diffusion rates of solutes, packed-bed geometry, etc. Pore size effects, pH, and other factors are not treated by these theories. Pore size effects in reversed-phase chromatography[40] have recently been studied. These results indicated that surface diffusion along the pore wall and restricted diffusion of small solute molecules in narrow pore regions influence band dispersion significantly. Rapid surface diffusion of ionic macromolecules along the pore walls of ion exchange packings is unlikely, but restricted diffusion should be a significant factor. Pore size effects have been studied in ion-exchange chromatography[41] of both small molecules and proteins with the conclusion that a 300-Å pore size yields higher resolution and loading capability for protein solutes than 100-Å and 500-Å packings. From the data compiled by Ginazza and Righetti[42] on protein size and charge distribution, it must be concluded that a nominal pore size of about 300 Å is an appropriate value to use for ion-exchange packings. Silica with a pore diameter of approximately 300 Å was employed in the synthesis of prototype packings. The 150-Å pore size materials were also included but only to study pore size effects.

Recently, ion-exchange processes have been examined in detail[43–48] with proteins as solutes. Barford *et al.*[43], using a number of supports, observed that elution patterns of proteins were inconsistent with accepted ion-exchange theory. Significant peak skew was observed. More recently, Kopaciewicz *et al.*[46] have developed a non-mechanistic model, which relates retention (k') to the number of charges involved in the adsorption/desorption process.

One obstacle to the study of solute band dispersion in any chromatographic process is peak skew. Theoretical models, such as those proposed by Knox[39] and others, which quantitatively describe band broadening due to axial diffusion, resistance to mass transfer in the mobile and stationary phases, etc., are based on the assumption that eluted peaks have Gaussian distributions. No detailed study of peak profiles of eluted proteins in ion-exchange chromatography with attention to skew can be found in the literature. The problem of peak skew was studied by Guiochon

and co-workers[49,50] in gas chromatography. They found that the shape of the asymmetric profile could be explained by the interaction of solutes with the curvature of non-linear isotherms. Barford *et al.*[43] had suggested this possibility earlier for the ion-exchange chromatography of proteins. This obstacle limits the scope of some of the results obtained of experiments designed for the preparation of this paper, since plate height measurements on non-Gaussian peak profiles are only approximate, at best. Essential chromatographic parameters, such as Knox constants (A,B,C)[39], are difficult to estimate under these conditions.

Surface charge–charge interactions with protein solutes: the Regnier model

The model of Kopaciewicz *et al.*[46] was used to investigate charge–charge and other interactions between protein solutes and the surfaces of siliceous and organic-based ion-exchange packing materials.

Retention can be described by the following expression:

$$k' = \frac{K_z}{(X^{+u}\,Y^{-v})^{2z}} \tag{1}$$

Where $k' = (t_R - t_0)/t_0$, K_z is a constant and z is the number of charges interacting between the solute and the surface; t_R is the retention time, and t_0 is the time required for an unsorbed solute to pass through the column after exploring the pore structure within the particles; X^{+u}, Y^{-v} represent the concentrations of the displacing ion and its counter-ion. The identities of these are reversed, depending on whether cation- or anion-exchange processes are involved.

In logarithmic form, eqn. 1 becomes:

$$\log k' = 2z \log[1/(X^{+u}Y^{-v})] + \log K_z \tag{2}$$

For the case of monovalent displacing and counter-ions, this expression predicts that,

TABLE I

LIST OF COLUMNS USED IN ION-EXCHANGE STUDIES

Designation	*Column*	*Ionic functional group**	*Pore size (Å)*	*Column length (cm)*	*Column I.D. (mm)*
A	Bio-Series SCX-150	$-SO_3H$	150	8	6.2
B	Bio-Series WCX-150	$-COOH$	150	8	6.2
C	Bio-Series WAX-150	$-CH_2NH_2$	150	8	6.2
D	Bio-Series SAX-150	$-CH_2NR_3$	150	8	6.2
E	Bio-Series SCX-300	$-SO_3H$	300	8	6.2
F	Bio-Series WCX-300	$-COOH$	300	8	6.2
G	Bio-Series WAX-300	$-CH_2-NH_2$	300	8	6.2
H	Bio-Series SAX-300	$-CH_2-NR_3$	300	8	6.2
I	Mono S	$-SO_3H$	800	5	5
J	Mono Q	$-NR_3$	800	5	5

* $R = CH_3$.

TABLE II

CATION EXCHANGE: REDUCED DATA FROM REGNIER MODEL FOR PROTEINS AS A FUNCTION OF pH

Column	*Eluite*	*Slope, m*	*Intercept, log K_z*	*Correlation coefficient*	*Mobile phase pH*
A	Lysozyme	3.91	−2.53	0.9960	5.40
		3.67	−2.19	0.9977	5.97
		3.87	−2.17	0.9978	6.49
		3.57	−1.80	0.9935	7.01
	RNAse A	4.97	−5.11	0.9970	5.40
		3.92	−4.44	0.9997	5.97
		3.76	−4.17	0.9996	6.49
		2.82	−2.99	0.9790	7.01
B	Lysozyme	2.72	−0.858	0.9790	5.40
		3.60	−1.67	0.9938	5.97
		4.41	−2.57	0.9990	6.49
		4.41	−2.78	0.9994	7.01
	RNAse A	4.86	−4.44	0.9901	5.40
		4.53	−4.33	0.9992	5.97
		4.36	−4.30	0.9986	6.49
		3.89	−3.96	0.9993	7.01
I	Lysozyme	5.69	−3.15	0.9998	5.40
		5.25	−3.32	0.9989	5.97
		5.15	−3.45	1.0000*	6.49
		4.75	−3.20	0.9994	7.01
	RNAse A	7.11	−6.37	0.9996	5.40
		5.32	−5.46	0.9997	5.97
		4.79	−5.23	0.9997	6.49
		4.37	−5.02	0.9997	7.01

* Two-point determination.

TABLE III

CATION EXCHANGE: REDUCED DATA FROM REGNIER MODEL FOR PROTEINS AT pH 6.4

Column	*Eluite*	*Slope, m*	*Intercept, log K_z*	*Correlation coefficient*
E	Lysozyme	3.38	−0.679	0.9970
	RNAse A	3.43	−2.27	0.9960
F	Lysozyme	4.52	−1.69	0.9957
	RNAse A	4.97	−3.37	0.9986
I	Lysozyme	5.09	−2.08	0.9920
	RNAse A	5.41	−3.90	0.9970

TABLE IV

ANION EXCHANGE: REDUCED DATA FROM REGNIER MODEL FOR PROTEINS AT pH 6.4

Column	*Eluite*	*Slope, m*	*Intercept, log K_z*	*Correlation coefficient*
H	Ovalbumin	5.54	−2.13	0.9975
	Conalbumin	3.65	−3.40	0.9983
D	Ovalbumin	4.69	−1.33	0.9954
	Conalbumin	3.14	−2.85	0.9997
J	Ovalbumin	6.13	−3.45	0.9956
	Conalbumin	3.31	−3.64	0.9224

when plotted against the reciprocal of molar concentration, the logarithm of retention data points will fall on a straight line of positive slope with a magnitude of $2z$. An extrapolation of this line will intersect the ordinate, and this value will be log K_z. For our purposes, namely, comparing different charged surfaces with respect to their protein sorption/desorption interactions, the absolute slope m, ($m = 2z$), was used, since the pH and salts which comprised the buffered mobile phases were identical in each case. Table I lists the columns used in this study.

A series of experiments was devised (utilizing the Regnier model as a theoretical basis) to study the chromatographic behaviour of several proteins on ion-exchange columns. The object of this effort was to compare siliceous, charged packings with their organic counterparts in ion-exchange chromatography. In principle, these respective surfaces could be expected to be vastly different in performance, the or-

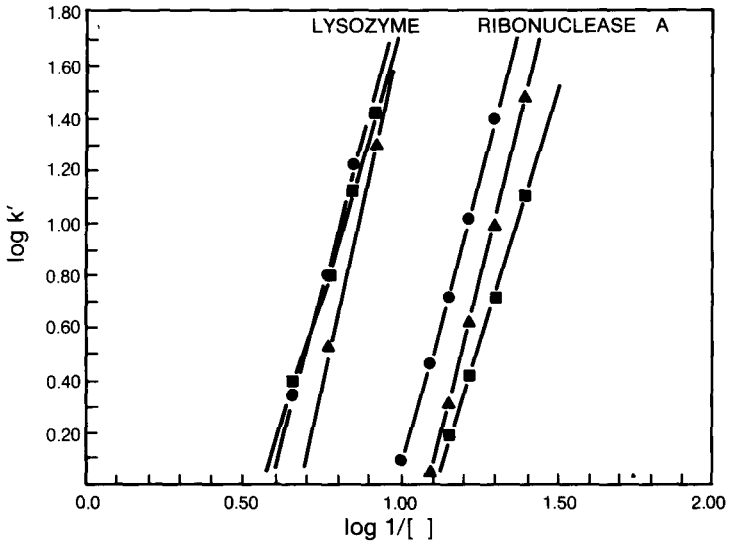

Fig. 1. Plot of log *k' versus* log 1/[ ] ([ ] = molar concentrartion of ammonium sulfate) for protein probes lysozyme and ribonuclease A. (■) Column A, (●) column B, (▲) column I. Mobile phase: 0.10 *M* potassium dihydrogen phosphate, ammonium sulfate (varied), 10 mg/l sodium azide, (pH 6.49). Flow-rate: columns A and B, 4 ml min^{-1}, column I, 2.5 ml min^{-1}. Ambient temperature.

ganic substrate introducing hydrophobic interaction phenomena, and the inorganic substrate producing silanophilic interactions. Cation-exchange columns were studied first, using the proteins ribonuclease A and lysozyme as surface probes. These substances are quite basic in nature. Lysozyme, in addition, is hydrophobic. If these substances are utilized as surface probes, then any secondary effects might be detected by their respective contributions to retention in each case. Tables II–IV show the results obtained in this study. Table II shows the results for the two proteins on two diol-modified ion-exchange packings (Bio-Series SCX-150, WCX-150), designated as columns A and B, respectively, and the Pharmacia Mono S material, designated as column I. Fig. 1 shows a plot of some of these data, calculated according to the model. It is remarkable that these three columns all produce very similar results, even though they differ significantly in composition, pore size, bonding processes, and charge densities. These parameters appear to have less influence on protein solutes than on small molecules in ion-exchange chromatography.

We next studied retention as a function of pH. Some of these results are plotted in Fig. 2 for column B (WCX-150). The pH is varied from 5.4 to 7.0 in cation-exchange chromatography, and retention decreases as the pH increases while, m changes only slightly. The value of m for lysozyme was plotted against pH, as shown in Fig. 3. Several effects can be noted in this plot:

(1) for column A, Zorbax Bio-Series SCX-150 (functional group $-SO_3^-$), the value of m changes only slightly as the pH increases.

(2) For column I, Pharmacia Mono S (functional group also $-SO_3^-$), the same trend can be noted with slightly greater dependence on pH.

(3) The value of m for column B, Zorbax Bio-Series WCX-150 (functional group –COOH) increases up to *ca.* pH 6.5 and then remains constant. If the value of m is directly related to charge–charge interactions, then increasing the mobile

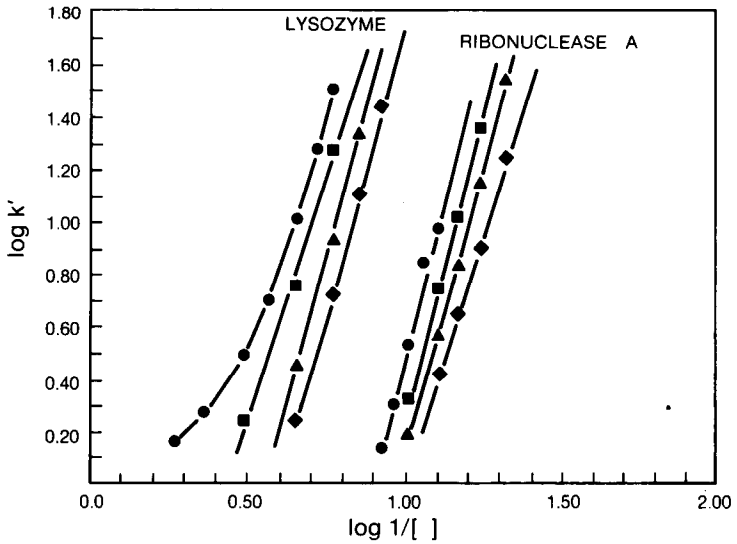

Fig. 2. Plot of log k' *versus* log 1/[ ] ([ ] = molar concentration of ammonium sulfate) for protein probes lysozyme and ribonuclease A on column B at pH 5.4 (●), pH 5.97 (■), pH 6.49 (▲) and pH 7.01 (◆). Other conditions as in Fig. 1.

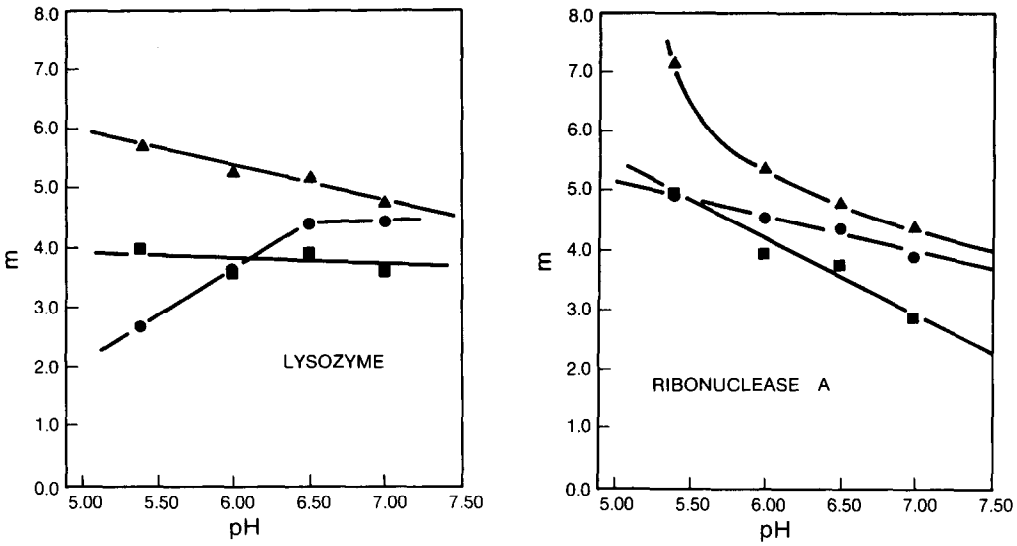

Fig. 3. Plot of *m versus* pH for protein probe lysozyme. (■) Column A, (●) column B, and (▲) column I.

Fig. 4. Plot of *m versus* pH for protein probe ribonuclease A. Other conditions and column identities the same as for Fig. 3.

phase pH would have the effect of decreasing the average number of protonated amino groups on the protein. In the case of the weak ion-exchange material, the increase in *m* up to pH 6.5 might be explained by charge density considerations, since ionization of the ligand functional group increases with increasing pH in this region. Another observation from Fig. 3 is that the magnitudes of the slopes for columns A and I, which both possess fully ionized ionogenic groups in this pH range, differ by more than one unit under these conditions. According to the model, a different average number of protonated amine groups on the protein are participating in the charge–charge interactions.

Fig. 4 is a plot similar to Fig. 3, except that ribonuclease A is utilized to probe the surface. This plot shows a similar pH influence on *m* for column I, but the effect is reversed for column B, because now the value of *m* decreases while pH increases. In order to inspect the influence of pH on log K_z, plots of these parameters were generated (not shown) from the data in Table II. In several instances, log k_z was seen to vary linearly with pH.

In other cases, *e.g.*, Mono S, K_z was noted to be rather insensitive to pH for lysozyme or followed the general linear trend for ribonuclease A that was observed for the other packings. Since the correlation coefficients of these plots are greater than 0.99 for 26 out of 28 cases, as shown in Table II, these data are sufficiently precise to state that the interactions between these charged surfaces and ionic macromolecules are quite complex. The data in Tables III and IV indicate that the slope (*m*) is usually greater for the organic packing than for its siliceous counterpart at pH 6.4 in anion-exchange chromatography. As in cation-exchange chromatography, this

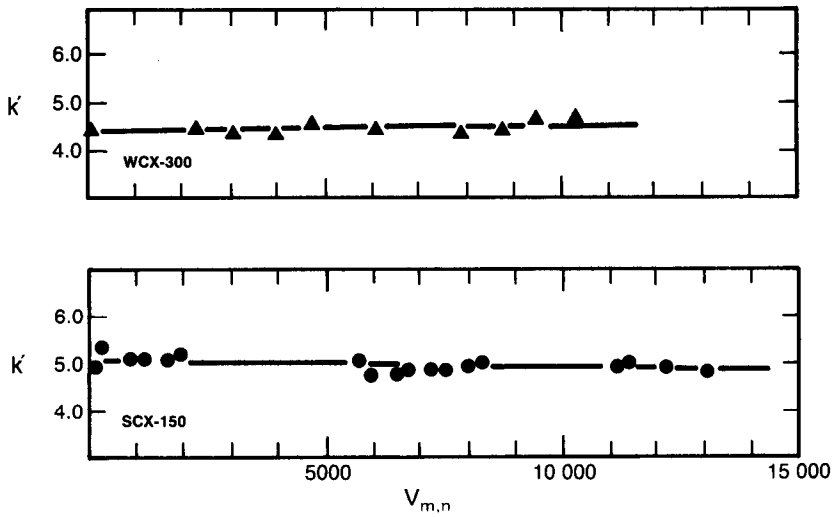

Fig. 5. Bonded phase stability test of cation-exchange columns at pH 8.25: retention (k' of lysozyme) *versus* number of void volumes ($V_{m,n}$). (●) Column A, (▲) column B. Mobile phases: column A, 0.05 *M* Tris, 0.09 *M* ammonium sulfate, 10 mg/l sodium azide (pH 8.25). Column B, 0.05 *M* Tris, 0.20 *M* ammonium sulfate, 10 mg/l sodium azide (pH 8.25).

must indicate that more charges are involved in the surface interactions for the organic packings.

The Regnier model, in its present form, does not completely describe the experimental observations and may need to be expanded to include pH as an additional variable.

Bonded phase stability tests

Bonded phase stability tests on the cation-exchange prototypes were conducted in a manner very similar to that reported earlier for the gel chromatography materials[33]. In that case $V_{m,n}$ is the number (n) of column void volumes (V_m) eluted during the test where V_m is equal to 1.8 ml, and n ranges from 0 to about 12 000 in each case. Chromatographic conditions were selected such that the retention of a suitable test probe (lysozyme) was in the range $4 < k' < 5$. The results of some of these stability tests are shown in Fig. 5. These tests were performed at a flow-rate of 1.0 ml min^{-1} (1440 ml of mobile phase or 800 void volumes per day) and ambient temperature.

An accelerated degradation test could have been devised for the test columns to reduce the length of time (2–3 weeks) required for each experiment, but this approach was not pursued. It was felt that the contact time between the flowing mobile phase and stationary phase was important, and that an experiment which closely approximates standard operating conditions would yield the most reliable results. A buffer of pH 8.25 ± 0.05 was chosen because this level has induced bonded phase hydrolysis for other column packing materials[33] at a rate which could be conveniently measured. This test differed somewhat from the previous gel chromatography experiments in that k' is large (*ca.* 4.5). Retention in ion-exchange chromatography depends on ligand density for charge–charge interactions and may also depend on

TABLE V

ELEMENTAL ANALYSIS OF COLUMN PACKINGS USED IN BONDED PHASE STABILITY TESTS

Column	*Initial carbon content**	*Final carbon content**	*% Carbon lost*
A	3.67, 3.52	3.52, 3.70	–
F	4.31, 4.31	4.31, 4.32	–

* Duplicate measurements.

silanophilic phenomena, since silanol groups are regenerated at the expense of the hydrolyzed bonded phase ligands. A basic, hydrophobic molecule, such as lysozyme could conceivably be maintained at constant retention by a balance of these two effects. For this reason, the columns were sacrificed and the packing material analyzed for elemental composition and compared to the composition of the starting material. These results are shown in Table V. The negligible change in carbon levels from the start to the end of the experiments indicates that the bonded phase is highly stable under these conditions. The apparent zero degradation is perhaps unrealistic, since these results are subject to the standard error of the carbon measurement, which is probably ±3–5% (relative).

CHROMATOGRAPHIC RESULTS

The results of the experiments described above prompted us to investigate the chromatographic behavior of these ion-exchange packings in detail in two areas: practical applications and theoretical considerations. Experiments were specifically designed to provide data from gradient elution conditions for correlation with non-linear-solvent-strength systems[51], a general chromatographic model[52], optimization of gradient elution conditions[53], and for this study.

Fig. 6 shows anion-exchange results with two protein samples for three columns: G, Zorbax Bio Series WAX-300; H, Zorbax Bio Series SAX-300; and J, Pharmacia Mono Q. The operating conditions were identical in each case. It can be seen that there are significant differences in retention and resolution among the chromatograms. The most notable difference is in the selectivity of the siliceous packing materials in their ability to resolve impure conalbumin (peak 2) into three peaks versus two for the organic packing. This resolution effect is not directly related to retention, since the Mono Q has the highest retention ($k' \cong 3$) for the doublet of the three peaks, while the best resolution can be observed with the SAX-300, which has the least retention ($k' \cong 2$). Also, the weak anion material (WAX-300) has greater selectivity and retention than the strong anion (SAX-300) material, even though the ligand density (μmole/m^2 of amine functional groups) of these two column packing materials is identical.

Studies were conducted with the cation versions of the diol-based packings. These results, shown in Fig. 7, are similar to those of the anion chromatography. α-Chymotrypsinogen is observed to have significantly different retention relative to the other protein eluates. It is more highly retained on Mono S ($k' = 6.4$). These

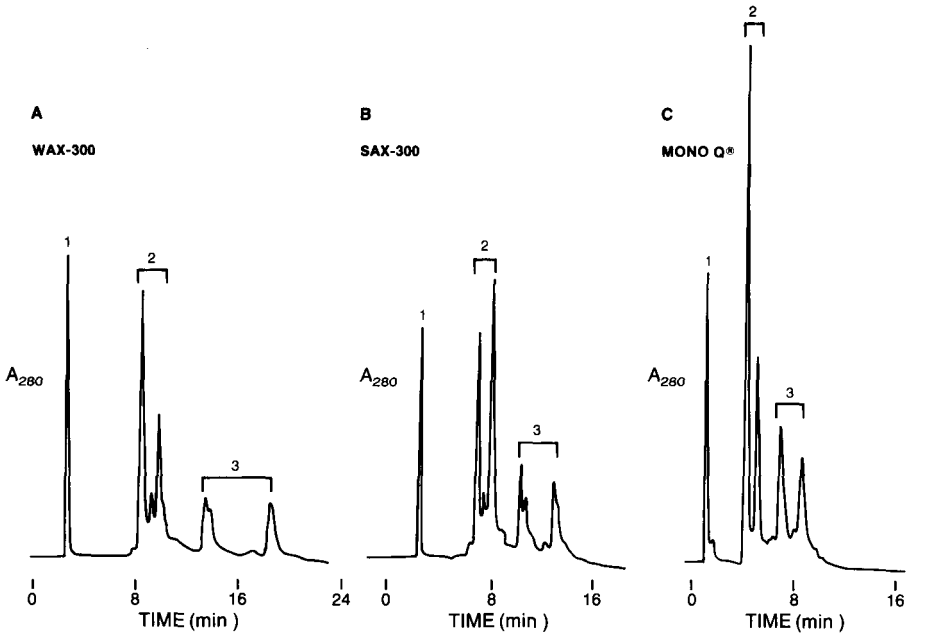

Fig. 6. Anion-exchange chromatography of conalbumin and ovalbumin. (A) Column G, (B) column H, (C) column J. Mobile phases: weak buffer, 0.02 M Bis-Tris, (pH 6.4). Strong buffer, 1.5 M sodium acetate, 0.02 M Bis-Tris, (pH 6.4). Gradient: 0–100% linear in 30 min. Flow-rate: 0.75 ml min^{-1}. Peak identities: 1 = uridine, 2 = conalbumin, 3 = ovalbumin.

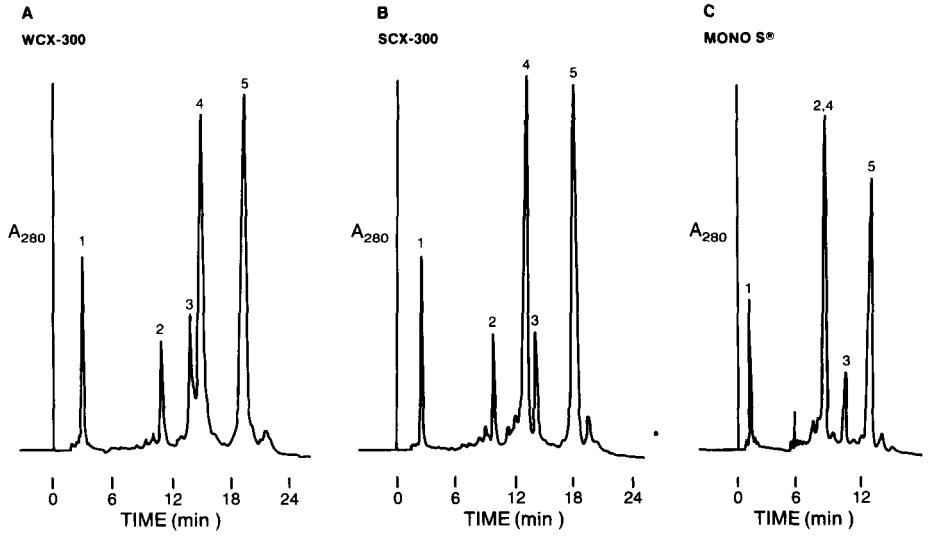

Fig. 7. Cation-exchange chromatography of basic proteins. (A) Column F, (B) column E, (C) column I. Experimental conditions are identical to those of Fig. 6. Peak identities: 1 = uridine, 2 = ribonuclease A, 3 = cytochrome *c*, 4 = α-chymotrypsinogen, 5 = lysozyme.

results are difficult to explain, since the siliceous supports have pore diameters of 300 Å with a specific surface area of 52 m^2/g, while the organic packing is believed to have a nominal 800-Å pore diameter with presumably much less surface area. The Regnier model clearly suggests that retention is directly proportional to the number of interaction sites on the matrix surface[46]. It could be postulated that the number of charge–charge interaction sites utilized by a given protein is not directly related to the ligand density of the packing material. It is also possible that hydrophobic interactions complicate the situation. Kopaciewicz *et al.*[46] added 1% of 2-propanol to the mobile phase at several different pH levels and compared the retention of several hydrophobic proteins to their retention in the absence of the organic additive. Their conclusion was that the organic substrate used by Pharmacia has little or no hydrophobicity. We performed a similar test for columns F and I (results not shown) and observed the same results with 1, 3 and 10% of 2-propanol, but this does not prove that such secondary equilibria are absent.

Application example: purification of mouse monoclonal antibody from ascites fluid

To compare the siliceous and organic-based ion-exchange packings, the purification of mouse anti-human tumor IgG was selected as an application example. A mobile phase system comprised of 0.02 *M* Bis-Tris, (pH 6.5) and 1.0 *M* sodium chloride was used in method development. The final chromatographic conditions were identical in each case, except for gradient slope (*e.g.*, 0–1.0 *M* sodium chloride for WAX-300 and 0–0.3 *M* for Mono Q). Fig. 8 A and B show analytical (30 µl

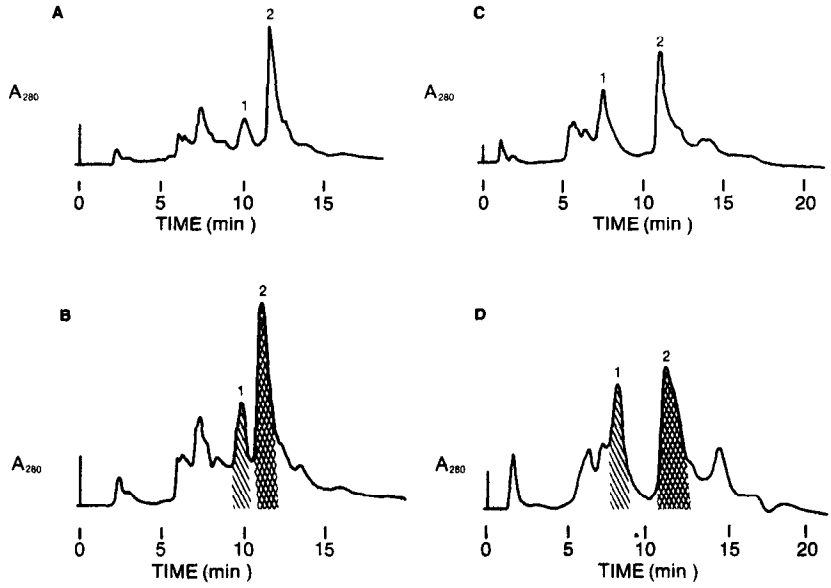

Fig. 8. Purification of mouse monoclonal antibody (IgG) from ascites fluid. (A, B) Column G, (C, D) column J. Mobile phase: weak buffer, 0.02 *M* Bis-Tris, pH 6.5. Strong buffer, 0.02 *M* Bis-Tris, 1.0 *M* sodium chloride, pH 6.5. Gradient: column G, 0–100% linear in 15 min. Column J, 0–30% linear in 15 min. Flow-rate: 1.0 ml min^{-1}. Peak identities: 1 = IgG, 2 = albumin. (Shaded areas indicate where effluent was collected.)

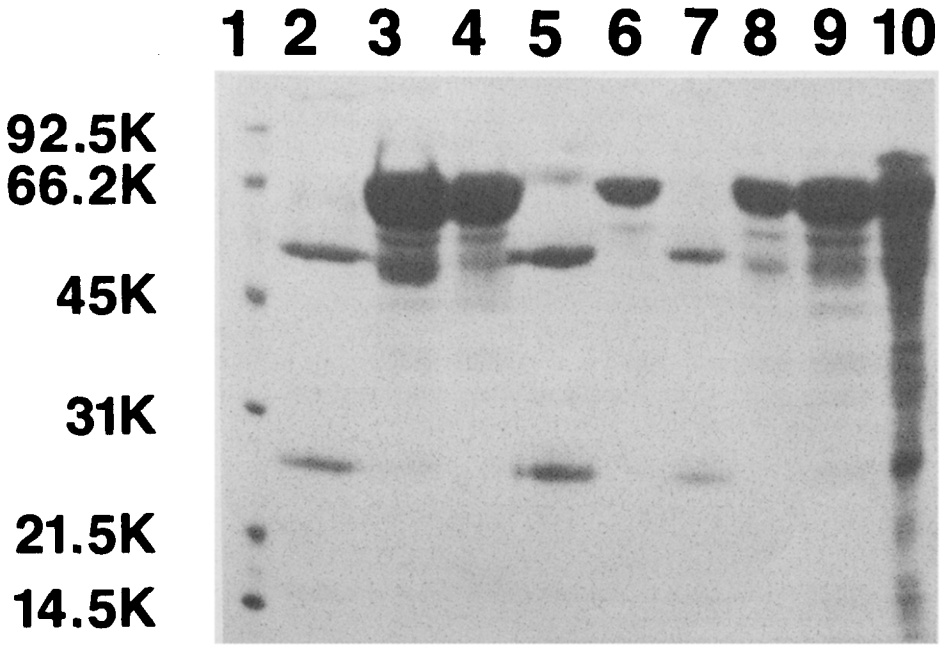

Fig. 9. Denaturing SDS–PAGE. Stain: Coomassie Brilliant Blue R-250. Lane 1: low-molecular-weight standards. Lane 2: IgG from fraction 1 of Fig. 8B. Lane 3: albumin and IgG from valley between fractions 1 and 2 of Fig. 8B. Lane 4: albumin from fraction 2 of Fig. 8B. Lane 5: IgG from fraction 1 of Fig. 8D. Lane 6: albumin from fraction 2 of Fig. 8D. Lane 7: duplicate of lane 2. Lane 8: duplicate of lane 6. Lane 9: albumin from fraction 2 of another run under identical conditions on Mono Q. Lane 10: untreated ascites fluid. K = kilodaltons.

injected) and "preparative" (200 μl injected) chromatograms for the WAX. Fig. 8C and D show results similarly obtained with the organic-based packing. Fractions were collected (shaded areas) from the effluent of each column and analyzed by denaturing SDS–PAGE. These results are shown in Fig. 9. Lane 1 shows the separation of low-molecular-weight standards and is used to calibrate the system. Lane 2 (WAX-300, fraction 1) should be compared with lane 5 (Mono Q, fraction 1). Under denaturing conditions IgG dissociates into two heavy chains (MW $\approx$ 50 000) and two light chains (MW $\approx$ 27 000). Lane 7 is a duplicate of lane 2. Albumin is the major component in ascites fluid and this substance can be seen in lane 3 (WAX-300, fraction 2) and lane 6 (Mono Q, fraction 2). These results should be compared to those of lane 10, which shows untreated ascites fluid. Apart from a difference in gradient conditions, the two columns give equivalent results under these experimental conditions.

SUMMARY AND CONCLUSIONS

A set of ten ion-exchange columns was subjected to a series of experiments in order to compare the chromatographic performance of siliceous *versus* organic ion-exchangers. The siliceous packing materials were prepared from a zirconium oxide-stabilized support with a hydrophilic molecular monolayer covalently attached to the

matrix. The diol functionality of these supports (Zorbax Bio Series GF-250, GF-450) was derivatized with strong and weak ionogenic functional groups. The bonded phase stability was measured for the cation columns in long-term experiments with the result that no detectable loss of organic surface material could be observed.

The surfaces were tested with hydrophobic and/or basic macromolecules, following the Regnier model as a theoretical basis. The results were in approximate agreement with the model in most respects, but certain observations could not be explained. There is some evidence that the model may have to be expanded to describe these findings more adequately. The experimental results of this paper suggest that zirconium oxide-stabilized silica can be used to prepare an inert hydrophilic support for subsequent covalent attachment of ionogenic functional groups. These new packings materials function in the ion-exchange mode in a manner very similar to other packings based on organic synthetic polymers, although significant differences in retention and selectivity were observed.

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