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HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF PROTEINS USING AZA-ETHER BONDED SILICA-BASED PHASES

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SUMMARY

The use of wide-pore silica-based hydrophilic aza-ether bonded phases for the chromatographic separation of proteins under anion-exchange conditions was studied. Polyether silanes containing terminal morpholine or piperazine derivatives are synthesized for attachment to the silica surface and provide a flexible approach to bonded phase design. In one instance, a quaternized amine support may be prepared by further derivatization of the methylpiperazine bonded phase. The supports provide high-performance anion-exchange chromatographic separations of proteins using gradients of increasing salt content, *e.g.*, to 1.0 *M* sodium acetate at pH 7.0. The salt type and concentration can be varied to control protein retention while the buffer system used at pH 7.0 exerts a minimal influence on the separation. The anion exchangers may be reproducibly prepared and exhibit chromatographic retention stability at pH 7.5 for at least 2 months of operation. Acceptable capacity for protein on the bonded phase is demonstrated with high recovery of solute mass. The flexibility in anion exchanger design provides a probe of bonded ligand hydrophobic effects which can contribute in an undefined and deleterious manner to the desired ion-exchange separation. Taken together, these results provide a greater insight into the operating characteristics of anion exchangers, especially with regard to competing retention mechanisms.

INTRODUCTION

High-performance anion-exchange chromatography (AEC) continues to be a widely used technique in the analysis and purification of biopolymers. Bonded anion-exchange phases have usually incorporated a tertiary amine functionality attached to carbohydrate, silica or polymer supports[1]. Quaternary ammonium salts immobilized on supports demonstrate an advantageous pH-independent permanent charge, but phase characterization is more difficult as these strong anion exchangers are usually prepared directly from the bonded tertiary amine phases.

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Currently, two main approaches are taken in the design of bonded tertiary amine phases for anion-exchange high-performance liquid chromatography (HPLC) of biomacromolecules. First, in analogy with classical anion-exchange soft gel supports, the diethylaminoethyl (DEAE) ligand is attached to silica or hydrophilic polymers[2,3]. Several commercial columns of this type have been extensively used in life science applications[4–8].

A second more recent approach, developed by Alpert and Regnier[9], involves the adsorption and cross-linking of polyethyleneimine (PEI) polymers on microparticulate porous silicas. The synthetic procedure provides control of the number of surface polymeric layers and the degree of hydrophobicity (by virtue of the cross-linking agent used[10]). A variety of commercially available packings utilize this type of chemistry[11–14].

Recent work in this field has examined new applications[1], improved column stability[15,16] and the capability for faster (< 1 min) separations[17].

In this paper, we present results on new anion-exchange phases obtained by attaching a morpholine or piperazine functionality to a polyether silane followed by bonding to silica. The columns achieve high-performance protein separations and exhibit good reproducibility, stability and capacity for protein. In particular, this bonded phase approach allows a variety of amines to be attached to the ether ligand for a probe of both ionic and hydrophobic contributions by the attached amine to protein retention and separation. Hydrophobic retention of protein on the anion exchangers may be examined under high salt mobile phase conditions typically of use in hydrophobic interaction chromatography (HIC).

EXPERIMENTAL

Equipment

The gradient liquid chromatograph (Beckman, San Ramon, CA, U.S.A.) consisted of two Model 114M pumps, a Model 340 mixer and injector, a Model 165 variable-wavelength UV detector (set at 280 or 260 nm) and a Model 427 integrator. A gradient delay volume of 5.9 ml was measured and subtracted from all chromatographic data presented.

Chemicals and materials

Methyl iodide was obtained from Aldrich (Milwaukee, WI, U.S.A.). Analytical-reagent grade organic solvents were from J.T. Baker (Phillipsburg, NJ, U.S.A.) and HPLC-grade water was prepared in-house. α-Lactalbumin (α-LAC, Type III, from bovine milk), transferrin (TRANS, human), two grades of ovalbumin ("pure" OVA, grade VI, 99% pure; and "crude" OVA, grade III, 90% pure), soybean trypsin inhibitor (STI, Type I-S), cytochrome *c* (CYT, type VI, from horse heart), ribonuclease A (RNase, Type III-A, from bovine pancreas), lysozyme (LYS, grade I, from chicken egg white) and α-chymotrypsinogen A (CHTG, type II, from bovine pancreas) were obtained from Sigma (St. Louis, MO, U.S.A.). Adenosine, adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP) and adenosine 5′-tetraphosphate (ATeP) (all from equine muscle) and ammonium acetate, sodium acetate, sodium chloride, sodium sulfate, potassium orthophosphate, Tris, Bis-Tris and Bis-Tris-propane were also obtained from Sigma.

Column hardware (blanks, fittings, etc.) was supplied by Extrudehone (Irwin, PA, U.S.A.) and Valco Instruments (Houston, TX, U.S.A.). Vydac silica gel of particle diameter 5.5 μm, nominal pore size 300 Å and surface area 74 m^2/g (data obtained from the manufacturer) was obtained from Separations Group (Hesperia, CA, U.S.A.).

Analytical Spherogel CAA HIC columns (10 cm × 4.6 mm I.D.) containing 5-μm 300-Å pore diameter silica-based bonded ether packing for HIC were obtained from Beckman.

Synthesis

The anion-exchange silanes were synthesized in a three-step procedure representing a modification of the previously published polyether silane synthesis[18]. The quaternized amine phase was synthesized by reaction of the methylpiperazine bonded phase with methyl iodide in methanol following a published procedure[19]. Table I lists the anion-exchange ether-based stationary phases synthesized and used in this study. Bonding of the trialkoxysilanes to silica was accomplished in a manner similar to that used previously[18]. Elemental analysis for surface coverage was performed by Multi-Chem Labs. (Lowell, MA, U.S.A.). The precision of the percentage carbon data for a given phase was *ca.* 3% relative standard deviation (R.S.D.). A correction is made for 0.62% carbon found in the unbonded Vydac silica gel. Nitrogen analysis data proved less precise (*ca.* 10% R.S.D.), but were of some value in the interpretation of bonded phase coverage (the unbonded Vydac silica showed 0.05% nitrogen). In the calculation of surface coverage in Table I, we approximate an average reaction of all three ethoxy groups per silane molecule. The actual stoichiometry of bonding continues to be under investigation.

Chromatographic procedures

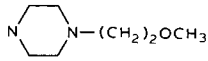
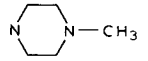
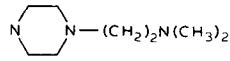
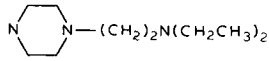
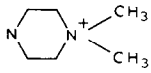
The anion-exchange phases were packed into 10 cm × 4.6 mm I.D. column tubes, following standard slurry procedures, using methanol as the driving solvent with a Model DSTV 122 air-driven pump (Haskel, Burbank, CA, U.S.A.). Mobile phases were prepared by adding the correct mass of salt and buffer to a volumetric flask containing HPLC-grade water, previously degassed by aspirator vacuum. The pH was adjusted to the desired value using phosphoric acid, glacial acetic acid, potassium hydroxide or ammonia solution, depending on the buffer system selection. Subsequently, a small amount of HPLC water was added to the mark. Using this procedure, mobile phase reproducibility in terms of the precision in protein peak elution volume on one column for the same sample mixture averaged 3.0% R.S.D.

RESULTS AND DISCUSSION

We describe here the successful separation of proteins by anion-exchange chromatography on bonded phases produced by reaction of any one of several aza-ether triethoxysilanes with the silica surface. Table I lists the variety of supports that were prepared in this work. In this approach, the aza-ether silanes, with one exception, were synthesized prior to bonding of the ligand to silica. This practice insures a high degree of bonded phase control and reproducibility. In one instance, a second reaction was performed on the bonded methylpiperazine (MP) phase with methyl iodide

TABLE I

CHARACTERISTICS OF ANION EXCHANGE PHASES, $\equiv Si(CH_2)_3(OCH_2CH_2)_nR$

Phase	*R*	*n*	*C(%)**	*Coverage** ($\mu mol/m^2$)*
MOR		2	4.17	4.7
MOEP		2	4.58	4.0
MP		2	5.42	5.7
DMAEP		1	2.25	2.0
DEAEP		1	3.47	2.8
QMP		2	5.37	5.2

* Percentage of carbon in bonded phase determined by elemental analysis, corrected for unbonded silica.

** Coverage is calculated assuming an average reaction of three ethoxy groups per silane molecule.

(see Experimental) to prepare the quaternized support (QMP). The bonded phase coverage is high at about 4.9 μmol/m^2, with the exception of the DMAEP and DEAEP phases, which possess bulky ligands on shorter spacers, at about 2.4 μmol/m^2.

The bonded phase design permits an increase in the number of nitrogen atoms per bonded ligand from one (morpholine, phase MOR) to two (methylpiperazine, phase MP) to three atoms (dimethylaminoethylpiperazine, phase DMEAP), and also a quaternized methylpiperazine phase (QMP). Lastly, the methoxyethylpiperazine (MOEP) and diethylaminoethylpiperazine (DEAEP) phases were prepared in order to examine the role of increased hydrophobicity on the bonded amine.

Fig. 1 shows the high-performance separation of four standard proteins on the MP phase at 25°C by use of a 20-min increasing salt gradient from a 10 m*M* potassium phosphate solution at pH 7.0 to the buffer solution containing 1.0 *M* sodium acetate. The separation of transferrin (TRANS, p*I* 5.5, mol.wt. 77 000), α-lactalbumin (α-LAC, p*I* 5.2, mol.wt. 17 500), ovalbumin (OVA, p*I* 4.7, mol.wt. 44 000), and soybean trypsin inhibitor (STI, p*I* 4.0, mol.wt. 21 500) occurs in order of decreasing p*I*, as expected. Note that sharp peaks are obtained with the resolution of a number of minor components. The profile obtained for OVA is similar to that obtained by others on DEAE- of PEI-type anion exchangers[20,21]. In fact, Fig. 2 illustrates that the separation obtained for OVA on the MP column is indicative of the sample purity. Fig. 2A shows the chromatogram obtained for a commercial ovalbumin sample of relatively low purity (*ca.* 90% OVA) and Fig. 2B that of the higher grade of OVA used in the separation in Fig. 1. The main peaks overlap well and indicate that

Fig. 1. Separation of standard protein mixture on MP phase. Standard proteins (1 = transferrin, 2 = α-lactalbumin, 3 = ovalbumin, 4 = soybean trypsin inhibitor) were chromatographed on a 10 cm × 4.6 mm I.D. MP column at a flow-rate of 1.0 ml/min using a 20-min linear gradient at 25.0°C from 10 m*M* potassium phosphate (pH 7.0) to 10 m*M* potassium phosphate (pH 7.0)–1.0 *M* sodium acetate. Detection, 280 nm, 0.1 a.u.f.s.; injection volume, 5 μl. Protein amounts: transferrin, 100 μg; α-lactalbumin, 25 μg; ovalbumin, 50 μg; soybean trypsin inhibitor, 50 μg.

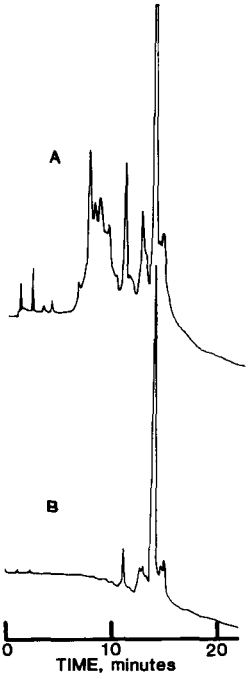


Fig. 2. Chromatography of two grades of ovalbumin on the MP phase. Chromatographic conditions as in Fig. 1. (A) Grade III, 90% protein, injection volume 5 μl, protein amount 50 μg; (B) grade VI, 99% pure protein, injection volume 5 μl, protein amount 50 μg.

TABLE II

COLUMN REPRODUCIBILITY OF MP PHASE

Mobile phase, A = 0.05 *M* ammonium acetate (pH 6.0), B = 0.05 *M* ammonium acetate (pH 6.0)–1.0 *M* sodium chloride, linear gradient from 0 to 100% B in 20 min; flow-rate 1.0 ml/min; detection at 280 nm, 0.1 a.u.f.s.; temperature, 25°C.

*Batch**	*C (%)*	V_g *(ml)***			
		TRANS	*α-LAC*	*OVA*	*STI*
1	4.87	2.8	4.9	6.6	8.6
2	5.09	3.3	5.2	7.2	9.1
3	5.39	3.4	5.2	7.2	9.0
4	5.42	3.5	5.5	7.6	9.3
Mean	5.19	3.3	5.2	7.2	9.0
R.S.D. (%)	5.0	9.6	4.7	5.8	3.3

* The four bonding batches were prepared separately using the same lot of silane and of silica.
** V_g = protein elution volume corrected for gradient delay volume.

the column will be of value in sample profiling and protein purification. Separate studies revealed that as much as 2 mg of protein may be loaded on to these columns with no indication of overloading (*i.e.*, no change in retention time)[22].

The column reproducibility and stability on any given aza-ether packing is good. For example, Table II presents column-to-column reproducibility data for the MP bonded phase. Each phase was prepared separately using the same batch of silane and Vydac silica and identical bonding reaction conditions. The carbon data show a precision of 5.0% R.S.D., which is an acceptable variation in the light of the 3% average precision of carbon analysis for one phase. The precision of the corrected (for gradient delay volume) protein chromatographic retention is found to agree from column to column within an average of *ca.* 5% for the well retained proteins, α-LAC, OVA and STI. The reproducibility of retention for TRANS is lower (*ca.* 10%), probably owing to the elution of this protein at the start of the gradient. Table III lists

TABLE III

STABILITY OF THE MP BONDED PHASE FOR PROTEIN AEC RETENTION

Conditions as in Table II.

*Volume of mobile phase passed (l)**	V_g *(ml)*		
	α-LAC	*OVA*	*STI*
0	5.5	7.6	9.3
8	5.0	7.3	9.1
16	4.4	6.7	8.8
20	5.0	6.6	8.7
Mean	5.0	7.1	9.0
R.S.D. (%)	9.0	6.8	3.1

* Mobile phase = 0.050 *M* ammonium acetate (pH 7.5)–0.2 *M* sodium chloride.

protein elution volumes on the MP phase as a function of use. The retention varies with an average precision of about 6% when 20 l of 50 m*M* ammonium acetate (pH 7.5)–0.2 *M* sodium chloride mobile phase are passed through the column. This column usage represents a 2-month period or *ca.* 500 samples injected. Fig. 3 shows chromatograms of the standard proteins obtained on a fresh MP column (Fig. 3A) and obtained after the passage of 20 l of pH 7.5 mobile phase (Fig. 3B). In Fig. 3B, less sample (4 μl) was injected than in Fig. 3A (6 μl). It can be seen that the two chromatograms are similar. Hence the aza-ether bonded phase chemistry is optimized for good column-to-column reproducibility and stability.

We next compared the relative chromatographic behavior of the four standard proteins used above on each of the anion-exchange supports listed in Table I. Table IV reveals a gradual increase in protein retention volume (V_g) in the bonded phase order MOR, MOEP, MP, DMAEP, DEAEP. The elution order of proteins on each column is as expected on the basis of anion exchange, *i.e.*, in order of decreasing p*I*. Interestingly, using the DMAEP bonded phase, the protein α-LAC failed to elute during the sodium acetate salt gradient, and neither α-LAC nor STI was recovered from the DEAEP column. Note that the quaternized anion-exchange QMP phase shows a similar retention to the DMAEP phase, but with elution of α-LAC.

The results in Table IV suggest that certain phases (DMAEP and DEAEP) exhibiting stronger anion-exchange protein retention may also show hydrophobic retention effects, resulting in poor recovery of α-LAC and STI. Recent studies have demonstrated the sensitivity of α-LAC retention to support hydrophobicity[23]. Moreover, the QMP phase, which is synthesized by addition of one methyl group to a nitrogen atom to form a permanent positive charge, exhibits anion-exchange reten-

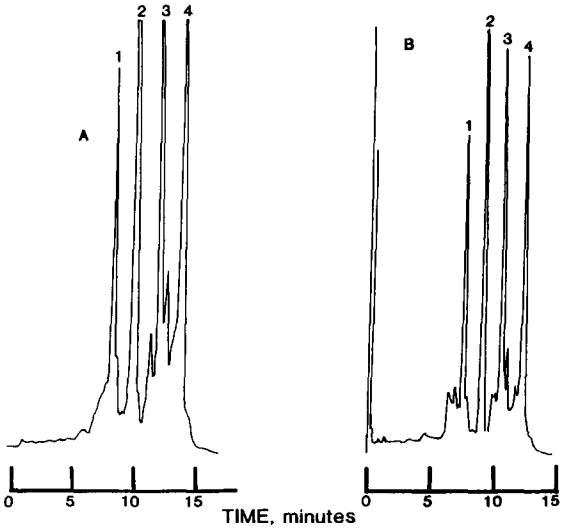


Fig. 3. Separation of standard protein mixture on the MP phase as a function of column usage. Chromatographic conditions as in Fig. 1, except the 20-min linear gradient was run from 50 m*M* ammonium acetate (pH 6.0) to the buffer plus 1.0 *M* sodium chloride. (A) Separation on a freshly prepared MP column, 6-μl injection of standard protein mixture. (B) Separation after 20 l of pH 7.5 mobile phase have passed through the column; 4 μl of standard protein mixture injected.

TABLE IV

COMPARISON OF AZA-ETHER BONDED PHASES FOR AEC OF PROTEINS

Mobile phase: A = 10 m*M* potassium dihydrogenphosphate (pH 7.0); B = 10 m*M* potassium dihydrogenphosphate (pH 7.0)–1.0 *M* sodium acetate. Other conditions as in Table II.

Phase	V_g *(ml)*			
	TRANS	*α-LAC*	*OVA*	*STI*
MOR	0	0	3.1	4.1
MOEP	2.2	3.0	4.7	6.5
MP	4.2	5.3	6.8	9.0
DMAEP	5.1	–*	7.5	11.9
DEAEP	6.7	–*	10.9	–*
QMP	5.2	6.5	7.5	11.3

* No elution observed.

tion as strong as the DMAEP phase but with good recovery of α-LAC. We have determined that all four of the standard proteins elute at V_0 (elution volume of unretained compound) on the ether HIC (with no bonded terminal amine) column under the ion-exchange conditions used in Fig. 1. Thus, phase hydrophobicity may arise from the terminal amine chosen. In the remaining sections of this paper, we examine in greater detail ion-exchange protein retention on these aza-ether columns and as the hydrophobic contribution of the terminal amine on the bonded phase to biopolymer retention.

ION-EXCHANGE EFFECTS

Although the protein chromatographic results in Table IV provide a ranking of the bonded phases in terms of anion-exchange strength, it is more valuable to have a quantitative measure such as the pK_a value of the bonded ligand. It was not possible to measure the pK_a of the bonded phase according to published methods[9] as slow equilibrium of the phase with the added titrant, even in 1.0 *M* sodium chloride solution, led to inconsistent results. We examined a chromatographic method using nucleotide retention following the procedures of El Rassi and Horvath[24]. Chromatographing the adenyl phosphate series on each of the aza-ether columns in Table I under isocratic conditions [10 m*M* potassium dihydrogenphosphate (pH 7.0)–0.1 *M* sodium acetate] yields a retention which is a function of the number of negative charges on the nucleotide. Fig. 4 shows a typical chromatogram of the series on the MP phase. The excellent performance achieved suggests that these columns may be of value in nucleic acid separations.

We had previously determined that on the uncharged ether bonded HIC column, all of the adenyl nucleotides elute at the void mark using these mobile phase conditions. Thus, a plot of log k' (k' = capacity factor) of the nucleotide *vs.* the charge on the solute can give a measure of the charge on each of the aza-ether bonded phases. Fig. 5 provides such a plot and Table V lists the slopes of the linear portions of the plots for each bonded phase. As may be observed, the ranking of the phases in order of increasing charge selectivity agrees well with the ranking of columns estab-

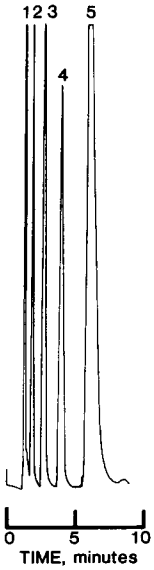


Fig. 4. Separation of adenyl nucleotides on the MP phase. Samples of nucleotides (1 = adenosine, 2 = AMP, 3 = ADP, 4 = ATP, 5 = ATeP) were chromatographed on the 10 cm × 4.6 mm I.D. MP column at a flow-rate of 1.0 ml/min using a mobile phase of 10 m*M* potassium phosphate (pH 7.0)–0.1 *M* sodium acetate at 25°C. Detection, 260 nm, 0.1 a.u.f.s.

lished previously for protein AEC retention (see Table IV), with the exception of the relative position of the QMP phase. The data in Table IV suggested that a significant hydrophobic contribution to protein retention existed on the DMAEP and DEAEP phases, which is not observed on the QMP phase. As may be seen in Fig. 5 and Table V, similar ion-exchange retention is obtained for sufficiently hydrophilic solutes, *i.e.*, nucleotides, on the QMP, DMAEP and DEAEP bonded phases.

For the columns that exhibit poor selectivity under these chromatographic conditions, a greater slope may be generated by the appropriate reduction in ionic strength, *e.g.*, MOR at 10 m*M* potassium dihydrogenphosphate with no sodium acetate present, or pH, *e.g.*, MOEP at pH 5.0 (see Fig. 5 and Table V).

Effect of pH

We next studied the influence of pH on the protein separations obtainable on the aza-ether columns. Table VI presents data for protein retention on the MP and QMP columns as a function of mobile phase pH. The phosphate buffer system employed has been shown to possess adequate buffer capacity for the pH range studied (6–8)[26]. In addition, all of the standard proteins should possess a net negative charge in this pH range. Similar retention values are noted at pH 6 and 7 on the MP column with a sharp decrease in retention at pH 8.0. The result suggests a loss of charge on the MP bonded ligand, i.e., $7 \leqslant pK_a \leqslant 8$. On the other hand, while retention on the QMP column fluctuates with pH, significant retention is still observed at pH 8.0. This relative independence of retention on pH is probably due to the permanent charge on the quaternized nitrogen of the QMP phase. The fluctuation in retention volume observed may indicate that only one nitrogen on the QMP phase is quaternized.

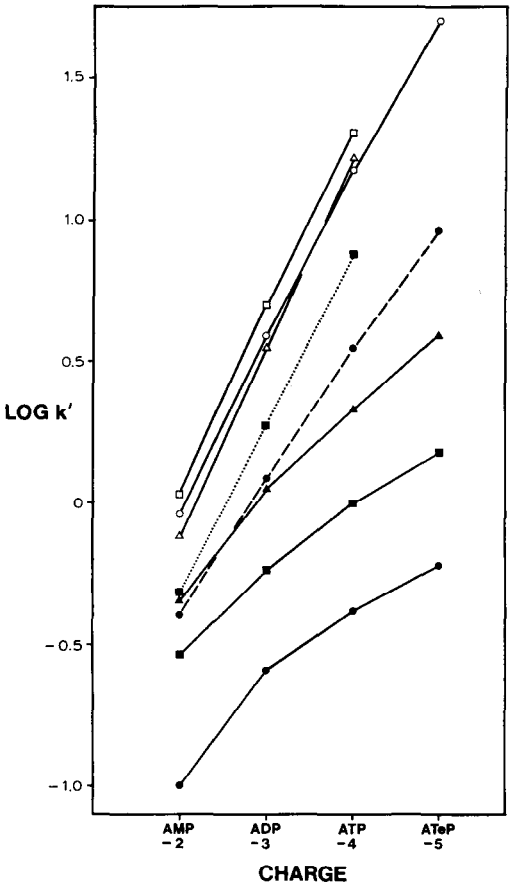


Fig. 5. Plot of log k' obtained on the aza-ether column *vs.* the charge number on the adenyl nucleotide series: adenosine 5'-monophosphate to adenosine 5'-tetraphosphate. Conditions as in Fig. 4 except (- - -) for MOR phase run in 10 m*M* potassium phosphate (pH 7.0) and (· · · ·) for MOEP phase run in 10 m*M* potassium phosphate (pH 5.0)–0.1 *M* sodium acetate. △ = QMP; □ = DEAEP; ○ = DMAEP; ▲ = MP; ■ = MOEP; ● = MOR.

TABLE V

SLOPE VALUES FOR LOG k' OF ADENYL NUCLEOTIDES *VS.* CHARGE NUMBER ON THE ADENYL NUCLEOTIDE AT pH 7.0

Mobile phase 10 m*M* potassium dihydrogenphosphate (pH 7.0)–0.1 *M* sodium acetate; flow-rate, 1.0 ml/min; temperature, 25°C; detection at 260 nm, 0.1 a.u.f.s.

Phase	*Slope*	*Phase*	*Slope*
MOR	0.18	DEAEP	0.641
MOEP	0.272	QMP	0.672
MP	0.270	MOR	0.453*
DMAEP	0.585	MOEP	0.60**

* Slope measured with an isocratic mobile phase of 10 m*M* potassium dihydrogenphosphate (pH 7.0).

** Slope measured with an isocratic mobile of 10 m*M* potassium dihydrogenphosphate (pH 5.0)–0.1 *M* sodium acetate.

TABLE VI

EFFECT OF MOBILE PHASE pH ON AEC OF PROTEINS

Mobile phase, A = 10 m*M* potassium dihydrogenphosphate (pH as indicated); B = 10 m*M* potassium dihydrogenphosphate (pH as indicated)–1.0 *M* sodium acetate. Other conditions as in Table IV.

Phase	*pH*	V_g *(ml)*			
		*TRANS (5.5)**	*α-LAC (5.2)**	*OVA (4.7)**	*STI (4.0)**
MP	6.0	2.7	4.3	6.2	8.9
	7.0	4.2	5.3	6.8	9.0
	8.0	0	0	0	1.9
QMP	6.0	3.2	4.4	6.0	9.9
	7.0	5.2	6.5	7.5	11.3
	8.0	3.6	5.3	5.7	9.9

* Values in parentheses are protein p*I* values from ref. 25.

The apparent loss of charge on the MP phase at pH 8.0 deserves discussion. Methylpiperazine has been found to possess pK_a values of 4.9 and 9.1 in aqueous solution[27]. The apparent reduction of the pK_a value of 9.1 by *ca.* 1.5 units in chromatographic practice may be explained in part by an examination of the bonded ligand structure. First, the methylpiperazine moiety is attached to the polyether "spacer" such that an oxygen atom is two atoms away from the ring nitrogen atom. pK_a values of 10.7 and 9.61 have been given[27] for ethylamine and 2-methoxyethylamine, respectively. Hence the influence of the added oxygen is to decrease the effective pK_a value, rendering the ligand less useful for anion exchange. In fact, the MOEP phase possesses ether side-chains on both piperazine nitrogens and exhibits less protein anion-exchange retention relative to the MP phase. The effect has also been noted for dextran-based DEAE phases[28] serving to decrease the pK_a value to 9.5.

Second, it is also possible that neighboring nitrogen atoms may have a significant influence on the pK_a of any one nitrogen as is observed for ethylamine (pK_a = 10.7) and ethylenediamine (pK_a = 9.9 and 6.8). Note, however, the influence of adding a third amine as in diethylenetriamine, with pK_a values of 9.0, 4.2 and 9.8, respectively. Finally, immobilization of the ligand on the silica surface may also influence its effective pK_a value.

Several routes exist to overcome this neighboring atom effect, including (1) the use of ligands with known high pK_a values, *e.g.*, DEAE; (2) increasing the distance between interacting atoms in the ligand chain and (3) quaternizing the amine to introduce a permanent positive charge. The second approach has been well studied by Alpert and Regnier[9] using PEI chemistry, *i.e.*, polymers with the repeating unit $(CH_2CH_2N)_n$. Insertion of nitrogen increases the bonded amine density and is preferable to longer alkyl chains between nitrogens, which increase the phase hydrophobicity. The DMAEP and DEAEP phases illustrate this approach. The use of quaternized ligands provides charge with less hydrophobicity as only a methyl group is added in the synthetic reaction. From the practical point of view, use of the MP phase at pH 8.0 would be preferable for the separation of strongly charged proteins, *e.g.*, p$I \leqslant 4.0$, while the QMP phase would effectively retain more weakly charged biopolymers.

Effect of salt

For the MP column, protein retention at pH 7.0 exhibited a minor dependence on any of several buffer salts chosen, including potassium phosphate, ammonium acetate, tris(hydroxymethyl)aminomethane (Tris), bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris), and 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris-propane). With the exception of ammonium acetate, all of these buffers show good buffer capacity at pH 7.0. The selectivity does not appear to vary greatly from buffer to buffer. Table VII indicates the influence of the eluting salt on protein retention using the MP column and the same 20-min linear gradient to 1.0 *M* salt concentration. Protein retention is greatest for sodium chloride, intermediate with sodium acetate and least with sodium sulfate. This order correlates well with the typical displacing ability of anions on solute retention observed in anion-exchange chromatography[29,30]. The relative retention of proteins is not greatly affected in each of the three salt systems, indicating that the salts have a general modulating effect on protein elution, in contrast to other reports[31]. It should be noted that the effect of salt on protein AEC retention in these experiments is opposite to their lyotropic (salting-out) ability, according to the Hofmeister series[32]. This observation is further evidence that the aza-ether columns achieve protein separation via anion exchange under these chromatographic conditions. Although we have used sodium chloride gradients in several of the separations throughout this work, in general we do not recommend its long-term use owing to the well known deleterious effects on stainless-steel pump surfaces[33].

Evaluation of nucleotide selectivity, pH and ionic strength effects on protein separation all indicate that the predominant mode of operation for these columns is anion exchange. However, relative protein retention behavior, as shown in Table IV, indicated that an apparent hydrophobic contribution to protein retention can occur when an amine of relatively greater hydrophobicity (*i.e.*, DMAEP and DEAEP) is selected for use as the bonded phase. We next examined stationary phase hydrophobicity attributable to the amine selected as the "active" ligand by use of the anion-exchange column in the HIC separation mode.

Hydrophobic effects

Previously, we had studied protein HIC retention on uncharged ether columns under high salt conditions[18]. Table VIII gives retention data obtained on the un-

TABLE VII

EFFECT OF ELUTING SALT TYPE ON AEC OF PROTEINS

Mobile phase, A = 10 m*M* potassium dihydrogenphosphate (pH 7.0); B = 10 m*M* potassium dihydrogenphosphate (pH 7.0)–1.0 *M* indicated salt. Other conditions as in Table IV.

Eluting salt	V_g *(ml)*			
	TRANS	*α-LAC*	*OVA*	*STI*
Sodium sulfate	2.7	3.5	4.0	5.7
Sodium acetate	4.2	5.3	6.8	9.0
Sodium chloride	4.6	5.8	7.3	9.2

TABLE VIII

COMPARISON OF MP AND ETHER PHASES FOR PROTEIN HIC

Columns, 10 cm × 4.6 mm I.D. containing either MP or ether phase; mobile phase, A = 3.0 *M* ammonium sulfate–0.5 *M* ammonium acetate (pH 6.0), B = 0.5 *M* ammonium acetate (pH 6.0), linear gradient from 0 to 100% B in 20 min; flow-rate, 1.0 ml/min; temperature, 25°C; detection at 280 nm, 0.1 a.u.f.s. CYT = Cytochrome *c*; RNase = ribonuclease A; LYS = lysozyme; CHTG = α-chymotrypsinogen.

Phase	V_g *(ml)*			
	*TRANS (5.5)**	*α-LAC (5.2)**	*OVA (4.7)**	*STI (4.0)**
MP**	9.5	10.1	8.8	11.6
Ether***	13.1	12.6	12.1	15.0
	*CYT (10.6)**	*RNase (9.4)**	*LYS (11.0)**	*CHTG (9.5)**
MP**	0	3.8	8.4	11.6
Ether***	4.9	9.6	12.1	16.7

* Values in parentheses are protein p*I* values from ref. 25.

** Bonded ligand is $\equiv Si(CH_2)_3(OCH_2CH_2)_2N$ (N—CH_3)

*** Bonded ligand is $\equiv Si(CH_2)_3O(CH_2CH_2O)_2CH_3$.

charged ether HIC column and the MP anion-exchange phase for eight proteins under HIC mobile phase and gradient conditions. Fig. 6 shows comparative chromatograms of the protein separations achieved on the two columns under HIC conditions. All of the proteins elute earlier on the MP phase relative to the ether HIC phase under the same mobile phase conditions. Thus, for all eight proteins, the MP phase is less hydrophobic than the uncharged ether column. Second, the elution order of

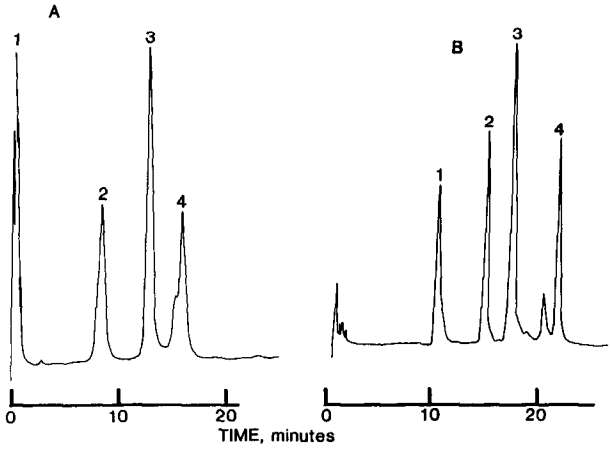


Fig. 6. Comparison of protein separation on MP and ether phases under HIC conditions. The sample of proteins (1 = cytochrome *c*, 2 = ribonuclease A, 3 = lysozyme, 4 = α chymotrypsinogen) were chromatographed on (A) the 10 cm × 4.6 mm I.D. MP column and (B) the 10 cm × 4.6 mm I.D. ether column [bonded ligand, $\equiv Si(CH_2)_3O(CH_2CH_2O)_2CH_3$] at a flow-rate of 1.0 ml/min using a 20-min linear gradient at 25.0°C from 3.0 *M* ammonium sulfate–0.5 *M* ammonium acetate (pH 6.0) to 0.5 *M* ammonium acetate (pH 6.0). Detection, 280 nm, 0.1 a.u.f.s.; injection volume, 5 μl. Protein amounts: cytochrome *c*, lysozyme and α-chymotrypsinogen, each 30 μg; ribonuclease A, 90 μg.

proteins with p*I* 4–6 is similar on both the ether and MP columns, with the exception of α-LAC, indicating largely HIC retention. The anomalous retention of α-LAC on the MP column suggests that ion exchange is still occurring. Fig. 6 supports this contention in that the earlier eluting peaks on the MP phase are broader than those eluting later on the ether column. It is well known in the RPLC of small molecules that multiple equilibria for retention can lead to broad peaks[26]. For proteins of p*I*>9, the elution order is similar for the two bonded phases, indicating an HIC separation mode. However, selectivity differences are evident, particularly in the front of the chromatogram (*i.e.*, CYT/RNase), suggesting that ion exchange may contribute to separation even under high salt mobile phase conditions.

Table IX lists more retention data for the proteins CYT, RNase, LYS and CHTG under HIC conditions on all six bonded aza-ether phases synthesized in this work. First, note that the protein elution order on all aza-ether columns matches that on the uncharged ether column, indicating a predominantly HIC separation mode. Second, the columns may be ranked in order of increasing protein HIC retention: MOR < MP < QMP < MOEP < DMAEP < Ether < DEAEP. This ranking is similar to that in terms of increasing protein AEC retention (Table IV) and that based on increasing nucleotide selectivity (Table V). This correlation further relates bonded amine hydrophobicity and ionic character. Hence the diethylaminoethyl portion of the DEAEP phase provides relatively greater AEC retention of proteins, but also greater HIC retention. The greater phase hydrophobicity is probably due to the diethyl chains of the DEAE ligand and results in poor recovery of α-LAC and STI.

Recent work has shown that similar effects operate in reversed-phase LC separations of proteins, *i.e.*, increased surface hydrophobicity by virtue of longer alkyl bonded chains[34] or increased bonded ligand density[35] leads to decreased protein recovery. From this point of view, the QMP phase provides the permanent charge needed for strong anion exchange with a minimum of added carbon, *i.e.*, a methyl group.

Thus, in the separation of proteins by ion exchange or HIC using bonded phase approaches, one must be aware of the properties of the bonded ligand, *i.e.*, ionic, hydrophobic, etc., which may contribute in an undefined and deleterious manner to

TABLE IX

COMPARISON OF AZA-ETHER BONDED PHASES FOR PROTEIN HIC

Conditions as in Table VIII.

Phase	V_g *(ml)*			
	CYT	*RNase*	*LYS*	*CHTG*
Ether	4.9	9.6	12.1	16.7
MOR	0	2.5	6.7	10.6
MP	0	3.8	8.4	11.6
MOEP	0	8.0	12.0	13.7
DMAEP	3.5	8.3	12.0	15.4
DEAEP	10.2	13.3	17.7	21.9
QMP	0	5.0	9.4	11.7

the desired separation mode for each solute. The concern is of special relevance with regard to the recent development of dual separation columns designed to perform in distinct separation modes using different mobile phase conditions[30,36–38].

CONCLUSIONS

The use of aza-ether columns for the high-performance AEC separation of proteins has been demonstrated. Hydrophilic polyether silanes containing terminal morpholine and piperazine derivatives may be synthesized for attachment to silica to provide a variety of bonded anion-exchange phases with a range of ionic and hydrophobic properties. In one instance, a quaternized support may be produced by further derivatization of the methylpiperazine phase. These aza-ether bonded phases are reproducibly prepared and exhibit acceptable stability and capacity. Protein separation, nucleotide selectivity and pH and ionic strength effects provide a probe of anion-exchange character on these phases. Protein separation by hydrophobic interaction under high salt mobile phase conditions indicates the hydrophobic character of the bonded amine ligand. It can be shown using these approaches that bonded amine ligand hydrophobicity is probably responsible for recovery losses of some proteins during AEC operation.

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