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STATIONARY PHASE CONTRIBUTIONS TO RETENTION IN HIGH-PERFORMANCE ANION-EXCHANGE PROTEIN CHROMATOGRAPHY: LIGAND DENSITY AND MIXED MODE EFFECTS*

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SUMMARY

Several anion-exchange stationary phases (based on polyethyleneimine-coated silica) were synthesized so as to vary in ligand density and hydrophobicity. These materials were first examined for hemoglobin-binding capacity and then evaluated chromatographically. Protein binding, retention and resolution increased concomitantly with ligand density. Ferritin (molecular weight 440,000) could not be eluted from the more highly-charged surfaces, but was desorbed from a low ligand density support. The above parameters also varied with the hydrophobic character of the stationary phase. Retention and resolution increased as more hydrophobic moieties were added. Data from a non-ionic hemoglobin-binding assay correlated reasonably well with anticipated matrix hydrophobicities. Possible explanations and applications of the observed phenomena are discussed.

INTRODUCTION

Ion-exchange chromatography has proven to be a valuable tool for the separation of proteins. The widespread incorporation of this technique into isolation schemes testifies to its versatility. Unfortunately, classical methods are laborious and time-consuming. Although the methodology has been frequently applied, little has been published regarding the underlying retention mechanism or factors which contribute to selectivity. The development of high-performance ion-exchange liquid chromatography allowed protein fractionations to be performed in less than 30 min. Fundamental studies on the ion-exchange process could now be completed within a reasonable period of time.

An understanding of the retention mechanism is essential to achieving chromatographic success. Kopaciewicz *et al.*[1] have recently proposed a model for the ion-exchange retention of proteins. In brief, this model suggested that only a fraction of the protein surface actually comes into contact with the chromatographic stationary phase, and that retention is exponentially related to the number of charges as-

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sociated with the adsorption–desorption process. Taking these hypotheses into consideration, experimental manipulation of stationary phase ligand density may have profound effects on chromatographic performance.

In addition, the environment surrounding the charged atom on the support may also modify the adsorption process, thereby influencing retention. Although the stationary phase charge is localized on a single atom (N in the case of anion-exchange), it must be remembered that these charges are located within matrices which may vary in porosity, hydrophobicity and dielectric constant. Hofstee[2] was among the first researchers to recognize the importance of hydrophobic contributions to ion-exchange protein chromatography. Research with a series of *n*-alkylamine-substituted (carbon chain length C_1, C_4 or C_8) agarose matrices suggested that protein binding resulted from a cooperative hydrophobic–electrostatic interaction. More recently, similar observations have been reported regarding the high-performance liquid chromatography of nucleic acids. Bishoff and McLaughlin[3,4] and Crowther and Hartwick[5] have synthesized support materials with both ion-exchange and hydrophobic properties for the separation of oligonucleotides. The unique selectivities obtained were thought to result from mixed ionic–hydrophobic retention mechanisms.

These reports have led us to examine stationary phase contributions to retention and resolution in high-performance anion-exchange chromatography (HPAEC) of proteins. Using the coating chemistry developed by Alpert and Regnier[6] for the preparation of silica-based macroporous anion-exchange matrices, we have synthesized and characterized several high-performance supports for protein separations. The stationary phases examined varied in either ligand density or surface hydrophobicity. These materials were first analyzed for ionic and non-ionic hemoglobin (Hb) binding. They were then evaluated chromatographically using myoglobin (MYO), ovalbumin (OVA), soybean trypsin inhibitor (STI) and ferritin (FER) as probes. Significant alterations in protein retention and resolution were seen. Chromatographic data were examined relative to changes in coating chemistry in an attempt to correlate physicochemical characteristics of the stationary phase to protein retention. The implications of these findings with regard to optimizing the chemical composition of HPAEC stationary phases are discussed.

MATERIALS AND METHODS

Silica

Vydac 101TPB 5.4 and 5.89 μm (spherical, 330 Å) silica was used throughout this study. It was a gift from The Separations Group (Hesperia, CA, U.S.A.).

Reagents

The following were purchased from Aldrich (Milwaukee, WI, U.S.A.): 1,2-epoxypropane, 1,2-epoxybutane, 1,2-epoxy-3-hydroxy-propane ("glycidol"), and 1,4-butanediol diglycidyl ether. Obtained from Polysciences (Warrington, PA, U.S.A.) were: polyethyleneimine-6 [molecular weight (MW) *ca.* 600], 1,3-diglycidylglycerol, triglycidylglycerol, and 1,2-ethanediol diglycidyl ether. The following reagents were products of Eastman-Kodak (Rochester, NY, U.S.A.): 1,2-epoxy-3-(*p*-nitrophenoxy)-propane, 1,2-epoxy-3-phenoxypropane, 1,2-epoxy-3-isopropoxy-propane, and 1,2-epoxy-3,3,3-trichloropropane.

Inorganic, reagents (for eluents and assays) and solvents (methanol, acetone, triethylamine, etc.) were AR grade or of comparable quality.

Proteins

Proteins used as chromatographic probes included the following: horse heart MYO (17,500; 6.9, 7.3), egg white OVA (43,500; 4.7), STI (20,100; 4.5), and equine FER (440,000; 4.3). Values in parentheses refer to MW and isoelectric point[7,8], respectively. All were purchased from Sigma (St. Louis, MO, U.S.A.) except FER which was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Bovine Hb (64,500; 6.8) from Sigma (crude powder, Type II; may contain up to 75% methemoglobin) was used for all Hb-binding assays.

Instrumentation

Two pumping systems were used for chromatographic evaluations: an LDC Constametric I and IIG system with a Gradient Master (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a Pharmacia FPLC instrument. Absorbance was monitored at 254 nm by a Model 150 or Model 153 Altex UV detector (Anspec, Ann Arbor, MI, U.S.A.). A Spectronic 70 (Bausch and Lomb, Rochester, NY, U.S.A.) was used to measure concentrations in the picric acid and Hb-binding assays.

Synthesis of packing materials

Coating and crosslinking. A modification of the method developed by Alpert and Regnier[6] was used. The directions which follow are for the synthesis of 1 g of material; the same component ratios were used in scale-up. An amount of 1 g of silica was suspended and thoroughly agitated in 10 ml of polyethyleneimine-6–methanol (w/v) solution of appropriate amine concentration. This mixture was allowed to stand at room temperature for 30 min. The polyamine-coated silica was then collected in a sintered-glass funnel and dried under vacuum for 30 min. Crosslinking was achieved by suspending the coated silica in 10 ml of a 10% (w/v) solution of the desired reagent in methanol. After 12 h at room temperature (without agitation) the mixture was heated on a steam bath for 1 h. The coated (crosslinked) silica was then filtered in a sintered-glass funnel, washed with methanol, dried under vacuum and stored in a dessicator. A range of ligand densities was obtained by varying the concentration of polyethyleneimine-6. Initial studies of ligand density and epoxide derivatization used a base coating crosslinked with 1,2-ethanediol diglycidyl ether. Other multifunctional epoxides (Table I) were later used following the same procedure.

Epoxide derivatization of crosslinked coating. An amount of 1 g of silica, coated and crosslinked as described above, was suspended in 10 ml of a solution of monofunctional epoxide–triethylamine–methanol (1:2:7, w/v/v). (Triethylamine catalyzes epoxidations[9].) The monofunctional epoxides used are shown in Table I. This mixture was allowed to stand at room temperature for 12 h and was then heated on a steam bath for 1 h. The derivatized, coated silica was then filtered in a sintered-glass funnel, washed with methanol, dried under vacuum and stored in a desiccator.

Elemental analyses (C, H, N) on selected coated silica samples were performed by H. D. Lee, Purdue University Department of Chemistry Microanalysis Laboratory.

Static analyses

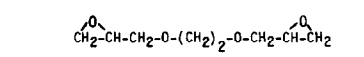
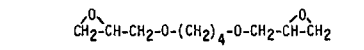
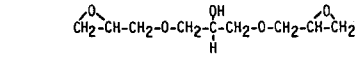
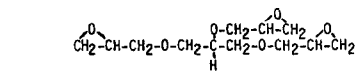
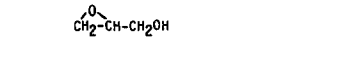
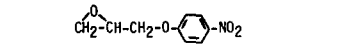
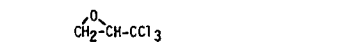
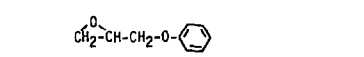
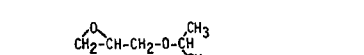
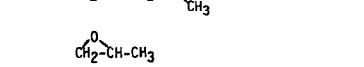
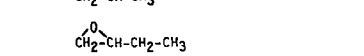
Picric acid ion-pairing capacity (IPC). Support ligand density, *i.e.* the sum of the non-ionized primary, secondary and tertiary amines present, was estimated using the picric acid assay described by Alpert and Regnier[6]. Samples of 50 mg each of coated silica were assayed.

Ionic hemoglobin-binding capacity (Hb_{iec}). A modification of the procedure developed by Chang *et al.*[10] was used. A 50-mg sample of coated silica was suspended in 2 ml of "adsorption buffer," *i.e.* 0.01 *M* Tris–HCl, pH 8.0, and equilibrated. Excess buffer was then removed by aspiration and 1 ml of Hb solution (5% w/v, crude bovine Hb in the adsorption buffer) was added to the test tube. The test tube was then vortexed and allowed to stand at room temperature for 15 min. At the end of this time, non-adsorbed Hb was removed by repeatedly washing the silica with the same buffer. Finally, Hb which had bound ionically to the silica was desorbed via the addition of 4–8 ml of a solution which consisted of 0.5 *M* NaCl in 0.01 *M* Tris–HCl, pH 8.0. This desorbed Hb (Hb_{iec}) was quantitated spectrophotometrically at 410 nm. (An absorbance of 1.0, using a 1.0-cm cell, corresponds to 0.13 mg/ml of Hb.)

Non-ionic hemoglobin-binding capacity (Hb_{iso}). In most cases, after repeated

TABLE I

MULTI- AND MONOFUNCTIONAL EPOXIDES USED FOR CROSSLINKING AND DERIVATIZATION

Reagent	*Abbreviation*	*Structure*
1,2-Ethanediol diglycidyl ether	EDGE	
1,4-Butanediol diglycidyl ether	BUDGE	
1,3-Diglycidylglycerol	DGG	
Triglycidylglycerol	TGG	
1,2-Epoxy-3-hydroxy-propane ("glycidol")	GLY	
1,2-Epoxy-3-(*p*-nitrophenoxy)-propane	ENPP	
1,2-Epoxy-3,3,3-trichloropropane	ETP	
1,2-Epoxy-3-phenoxypropane	EPP	
1,2-Epoxy-3-isopropoxypropane	EIP	
1,2-Epoxypropane	EP	
1,2-Epoxybutane	EB	

washes with the NaCl–Tris solution described above some Hb remained bound to the coated silica surface (as evidenced by its color). This residual Hb could not be quantitatively removed with even higher salt concentrations, but could be desorbed with a reversed-phase eluent [trifluoroacetic acid–water–isopropanol (1:400:600)]. This desorbed, non-ionically bound Hb (Hb_{iso}) was also measured spectrophotometrically.

The ratio of the two values for Hb-binding capacity (iec–iso) was used as a qualitative measure of relative stationary phase hydrophobicity.

Chromatographic evaluation

Portions of the coated silicas were packed into 5 × 0.41 cm I.D. columns[11] for evaluation as HPAEC stationary phases. Standard chromatographic conditions were a 20-min linear gradient from 0.01 *M* buffer ("A") to 0.5 *M* NaCl in 0.01 *M* buffer ("B") at a flow-rate of 1 ml/min. Retention mapping was performed as previously-described[1] using several proteins selected to cover a range of retention behavior. (Samples were prepared at pH 7.0.) A mixture of MYO, OVA and STI (3, 7 and 20 mg/ml, respectively) and FER (2 mg/ml) served as probes. Resolution was calculated for OVA and STI major peaks in the three-component sample [*i.e.*, $R_s = 2(t_{R^2} - t_{R^1})/(\Delta t_{R^1} + \Delta t_{R^2})$].

RESULTS AND DISCUSSION

Stationary phase synthesis

The objectives of this research were to examine the influence of support ligand density and matrix hydrophobicity on retention and resolution in HPAEC of proteins. Such an investigation required the synthesis of several anion-exchange stationary phases in which each variable was altered independently. The chemical reactions chosen to accomplish this task were similar to those described by Alpert and Regnier[6] in which polyethyleneimine (PEI) was electrostatically adsorbed to the surface of macroporous silica and crosslinked into a continuous film (Fig. 1). Once placed on the surface, stationary phase amines can be further modified with a variety of electrophilic reagents (assuming that crosslinking is controlled so as not to consume all reactive amines). Since this film is ionically adsorbed to the surface at many sites and covalently crosslinked, it is quite stable. As illustrated in Fig. 1, the polyamine is adsorbed to the silica surface via primary amines only. While steric considerations make this likely, it has not actually been proven.

Alpert and Regnier[6], Vanacek and Regnier[11] and Pearson and Regnier[12] have reported that the ligand density of the PEI-coated supports can be varied by (a) adjusting the concentration of PEI in the coating solution; (b) repeatedly washing the uncrosslinked film with methanol or (c) using different molecular weight fractions of PEI. The first method was used in these studies because of its reproducibility (± 5%) and ease of application.

The uncrosslinked PEI coating is composed of a population of primary, secondary and tertiary amines in what is thought to be a 1:2:1 ratio[13]. Subsequent modification with any electrophilic reagent (*e.g.* crosslinking) will shift the population of amines toward tertiary and modify the physical-chemical environment surrounding the amines. The nitrogens involved in the reaction can be affected in several ways.

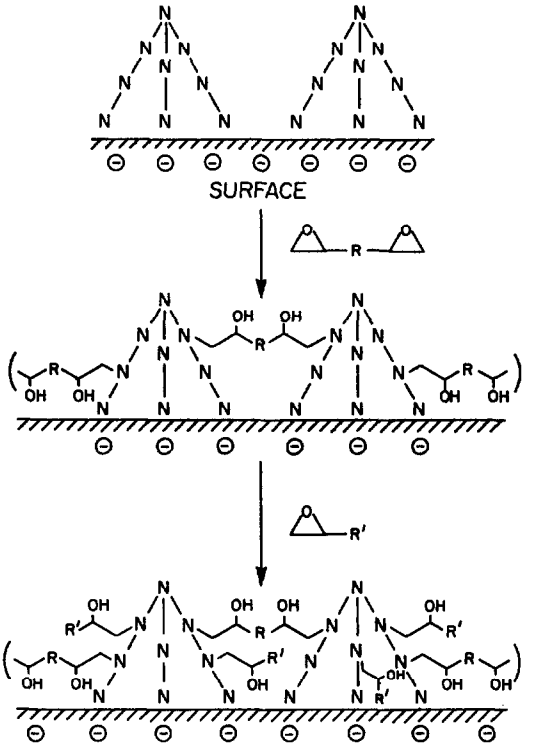


Fig. 1. The synthetic route to an adsorbed polyethyleneimine stationary phase. Structures have been oversimplified for illustration (*e.g.* amine molecules are depicted without ethylene groups or protons.) R and R′ denote variable parts of epoxides which may be used for crosslinking and subsequent derivatization (see Table I).

Depending on the electrophile used, hydrophobic sites and/or steric hindrance may be introduced. The dielectric constant associated with the stationary phase surface can also be altered[14]. Since these coatings are polyelectrolytes, lightly-crosslinked coatings may swell relative to those which are more extensively crosslinked. Thus, stationary phase dimensionality may be affected by the degree of crosslinking. The crosslinking step, in turn, can be controlled by reagent concentration, reaction time or the addition of a catalyst.

Evaluation of stationary phases

Two types of test methods were used to compare the support materials synthesized: (1) a static binding assay and (2) chromatographic evaluation.

Hemoglobin-binding assay. Hemoglobin has long been used to assess the relative binding capacities of these types of support materials[6,10]. Typically, Hb is bound at low ionic strength and desorbed by increasing the salt concentration (Hb_{iec} assay). Although not previously reported, it was noted that even repeated washes with relatively high salt (NaCl) concentrations failed to remove Hb bound to the matrix in some cases. However, quantitative desorption could be achieved with a reversed-phase eluent (trifluoroacetic acid–isopropanol; Hb_{iso} assay). Since an organ-

ic solvent was required, a hydrophobic-related mechanism is probably involved. Hofstee and co-workers[14,15] have suggested that cooperative ionic–hydrophobic interaction between proteins and chromatographic stationary phases may become significant when both the protein and the stationary phase are sufficiently hydrophobic. In their native state, proteins exhibit considerable diversity in this respect. Both bovine serum albumin and Hb appear to be relatively lipophilic[14–17]. Interestingly, in our studies, the non-ionic binding exhibited by Hb was not observed for bovine serum albumin suggesting that this phenomenon was more a function of Hb than of the support material. A specific interaction between Hb and the ion-exchange matrix may be involved. For this reason, Hb may not be the optimum probe for evaluation of static binding capacities. However, an attempt was made to correlate non-ionic Hb binding with the chemical character of the stationary phase. Since both the ionic and non-ionic Hb-binding capacities of these support materials varied, a ratio of Hb_{iec} and Hb_{iso} values (iec/iso) was used for comparisons.

Chromatographic evaluation. The chromatographic characterization of new protein-compatible ion-exchange matrices requires the examination of both retention and resolution. Since retention depends on the number of charge interactions and both the protein and support may ionize independently with respect to pH, retention was measured over the pH range 5–10. For each support, a "retention map"[1] was constructed with the dependent variable expressed as a percent of the desorption eluent, "B" (100% B = 0.5 *M* NaCl in 0.01 *M* buffer) for all proteins chromatographed. The independent variable, pH, and operation protocol were constants. Since the objective of these studies was to examine the influence of stationary phase variables, it was necessary to use as probes proteins which are relatively constant in their apparent charge across the pH range tested. Such proteins were identified through their chromatographic behavior on a quaternary amine strong anion-exchange column[18]. The charge density of such a support is independent of mobile phase pH, in contrast to a weak anion-exchange column, so that variations in retention result

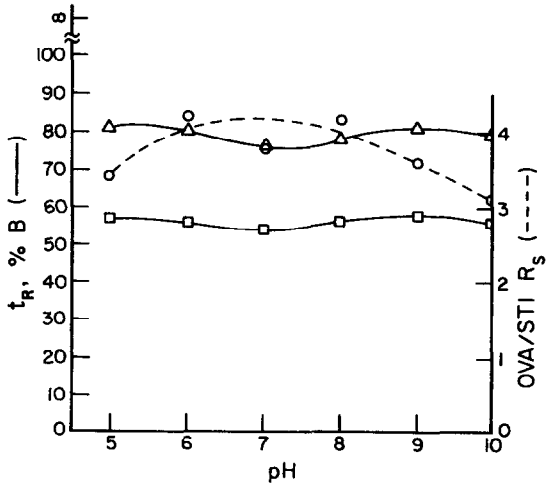


Fig. 2. Retention and resolution maps of STI and OVA chromatographed on a quaternary amine strong anion-exchange stationary phase. The retention of STI (△) and OVA (□) as well as R_s (○) were measured as a function of pH (see Materials and methods).

solely from charge alterations in the protein. STI and OVA met these requirements fairly well (Fig. 2). The retention behavior of these two proteins on the quaternary amine column was then used as a basis for interpreting retention maps constructed for all other stationary phases.

Snyder and Kirkland[19] have shown that for small molecules resolution increases with retention. In the case of protein ion-exchange chromatography, this may not always be true[20]. Plots of resolution *vs.* retention during gradient elution often show a maxima at intermediate values. Although not proven, such anomalous behavior probably results from a large kinetic contribution to band-spreading[21]. Therefore, adsorption–desorption kinetic contributions to band-spreading appear to be independent of simple mass transfer band-spreading components. These effects are variable depending on the elution conditions, protein probe and physicochemical composition of the stationary phase. At present, the retention-dependent contribution to resolution is unpredictable and must be determined experimentally. Thus, the pH dependence of resolution between OVA and STI was examined via the construction of "resolution maps" similar to those described for protein retention.

Ligand density

Anion-exchange stationary phases of varying ligand densities were synthesized

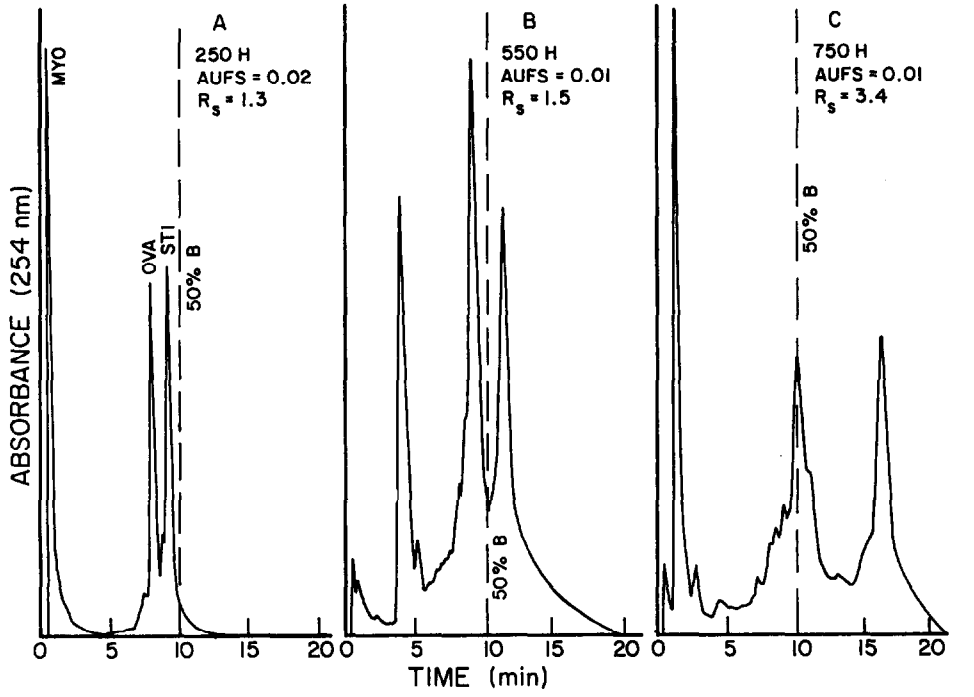


Fig. 3. The effect of stationary phase ligand density on protein retention. A three-component protein mixture (MYO–OVA–STI) was chromatographed on three supports of varying ligand density (IPC: 250, 550, 750 μmol N/g support; "H" denotes an underivatized coating). Chromatography was performed at pH 8.0, using a 20-min linear gradient from 0 to 0.5 *M* NaCl at a flow-rate of 1 ml/min. A dashed line has been included at the gradient midpoint to facilitate comparisons. Note the change in detector AUFS between (A) and (B,C).

TABLE II

EFFECT OF STATIONARY PHASE LIGAND DENSITY ON SELECTED PARAMETERS

PEI-6 (%)	*IPC (μmol N/g support)*	*Hb_{iec} (mg/g support)*	*t_R (% "B")**				*R_s** (OVA–STI)*
			MYO	*OVA*	*STI*	*FER*	
1	250	28	3	40	46	59	1.3
5	550	33	20	46	57	NE	1.5
15	750	44	3	51	81	NE	3.4

* Chromatography was performed at pH 8.0, with "B" eluent consisting of 0.5 *M* NaCl in 0.01 *M* Tris–HCl buffer. Proteins were eluted with a 20-min linear gradient from 0–100% "B" at a flow-rate of 1 ml/min (NE = not eluted).

** R_s calculated by the equation given in Materials and methods (*Chromatographic evaluation* section).

as described in Materials and methods. A three-fold range in ligand density, as determined by picric acid ion-pairing capacity (IPC), was obtained. High ligand density coatings exhibited favorable chromatographic characteristics for analytical protein separations (Fig. 3). Retention (t_R; expressed as % "B"), resolution (R_s) of the OVA–STI pair and Hb_{iec} all increased with increasing ligand density (Table II). It should be noted that even at low ligand densities, there may be a large excess of stationary phase nitrogens compared to the number of charged residues on a protein. However, up to 50% of these nitrogen atoms may be inaccessible[11] and only a small portion of the accessible amines may be ionized. Therefore, the actual charge on the support surface will be some fraction of the IPC value.

A disadvantage of the high ligand density coatings was that they gave lower recovery in some cases. FER could not be desorbed from either the intermediate or highest ligand density support, but did elute from the column having the lowest IPC value (Table II). Evidently, the low ligand density of this material decreased protein–stationary phase interaction. Since OVA–STI R_s on this column was substantially less than that on the highest ligand density support, FER recovery was not gained without sacrifice. A support which simultaneously exhibits high selectivity and good recovery of high-molecular-weight proteins may not be attainable.

Amine substitution

It has been shown that the addition of hydrophobic character to an ionic stationary phase significantly altered selectivity in oligonucleotide separations[3]. To determine if a similar approach could be used to modify the ion-exchange chromatography of proteins, several PEI-coated stationary phases varying in hydrophobicity (different R′ groups, see Fig. 1) were synthesized. Initially, portions of the lowest ligand density (IPC = 250 μmol N/g support) packing material were derivatized with one of two small monofunctional epoxides, glycidol (GLY) or 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (ENPP) (Table I). Titration of GLY-derivatized material showed an upward shift in the curve suggesting a higher ionization state (data not shown). The reaction product of GLY is a very polar, hydrophilic moiety, whereas ENPP provides a larger, more hydrophobic group. Both derivatized stationary phases exhibited notably lower Hb_{iec} values than the base material (Table

TABLE III

EFFECT OF POST-CROSSLINKING DERIVATIZATION

Derivative	*Hb_{iec} (mg/g support)*	*t_R (% "B")***				*R_s**** *(OVA–STI)*
		MYO	*OVA*	*STI*	*FER*	
None*	28	3	40	46	59	1.3
GLY	18	3	37	40	44	0.7
ENPP	15	3	41	56	NE	2.3

* Underivatized coating (crosslinked with EDGE) IPC = 250 μmol N/g support.

** Same chromatographic conditions as in Table II.

*** R_s calculated by the equation given in Materials and methods (*Chromatographic evaluation* section).

III). Retention and resolution were lower on the GLY derivative than on the underivatized support. In contrast, the ENPP derivative showed a relative increase in both chromatographic parameters (Fig. 4). FER was recovered from the GLY derivative, possibly as a result of decreased protein binding affinity; however, it did not elute from the ENPP column.

To investigate further these effects, additional PEI-coated silica was derivatized with one of five other monofunctional epoxides (Table I). (Picric acid assay of the "base material" indicated 280 μmol amine per gram of support. Elemental analysis of selected derivatized coatings showed 5–7 epoxide molecules reacting per PEI-6

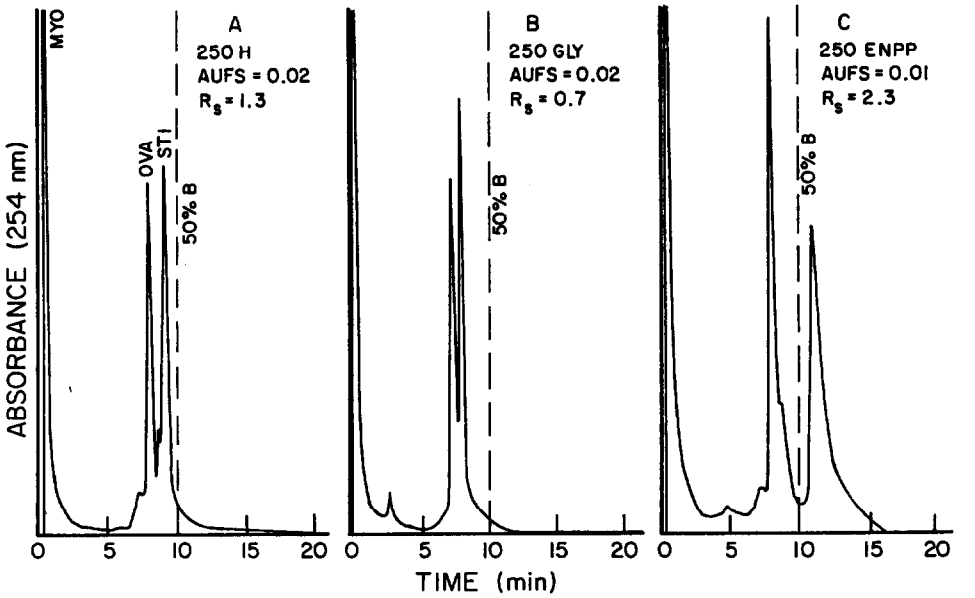


Fig. 4. The effect of post-crosslinking derivatization on retention and resolution. A three-component protein mixture (MYO–OVA–STI) was chromatographed on three stationary phases of equal ligand density, but varying surface hydrophobicity (250 GLY and 250 ENPP refer to epoxide derivatives of the base coating, "H"). Chromatographic conditions same as for Fig. 3. Note change in AUFS between (A,B) and (C).

TABLE IV

FURTHER INVESTIGATION OF STATIONARY PHASE AMINE SUBSTITUTION

Derivative	Hb_{iec}**	Hb_{iso}**	Hb_{tot}**	*iec/iso*	t_R (% "B")***				R_s§ (OVA–STI)
					MYO	*OVA*	*STI*	*FER*	
None*	16	16	32	1.0	4	39	48	78	2.1
ETP	3	21	24	0.1	5	38	61	NE	1.8
EPP	6	25	31	0.2	4	41	69	NE	2.3
EIP	12	11	23	1.1	3	35	40	47	1.3
EP	14	10	24	1.4	3	35	40	47	1.4
EB	16	14	30	1.1	3	38	45	69	1.6

* Underivatized coating (crosslinked with EDGE) IPC = 280 μmol N/g support.

** All values are mg Hb/g of support material; Hb_{tot} = total Hb-binding capacity.

*** Same chromatographic conditions as in Table II.

§ R_s calculated by the equation given in Materials and methods (*Chromatographic evaluation* section).

molecule, 14 N atoms, when the epoxide was GLY, EP or ETP, but only 2–3 epoxides per PEI-6 molecule when ENPP or EPP was used.) In order to analyze more specifically differences among the stationary phases produced, data from both ionic and non-ionic Hb-binding assays were utilized. Higher Hb_{iso} values and substantially lower iec/iso ratios (Table IV) were obtained with 1,2-epoxy-3,3,3-trichloropropane (ETP) and 1,2-epoxy-3-phenoxypropane (EPP). Although there was an increase in STI retention on both stationary phases (compared to the underivatized material) OVA–STI R_s was greater on the EPP column only. Since retention increased concomitantly with the hydrophobic character of the matrix a mixed ionic–hydrophobic mechanism is probably responsible[14,15].

The remaining three epoxides —1,2-epoxy-3-isopropoxy-propane (EIP), 1,2-epoxypropane (EP) and 1,2-epoxybutane (EB)— yielded stationary phases which were less hydrophobic than the underivatized material. Chromatographic data from the EIP and EP derivatives were indistinguishable (Table IV). Although EIP results in the addition of a moiety twice the size of that from EP, the chemical character of these groups should be similar. Because the substituent is not rigid, it may be able to move away from the interacting charged groups (protein and support) without affecting chromatographic performance. The EB derivative exhibited higher Hb_{iso} and t_R values than the EP derivative, suggesting a greater degree of non-ionic interaction. Whereas reaction of EP places an isopropanol group on the amine, reaction with EB adds a *sec.*-butyl alcohol moiety. *sec.*-Butyl alcohol ranks higher in the eluotropic series for reversed-phase chromatography and may be viewed as the more hydrophobic of these two substituents. All three of these support materials appeared to be more hydrophilic than the underivatized coating, since they showed decreased Hb_{iso} and t_R values. Apparently, the crosslinking reagent (1,2-ethanediol diglycidyl ether, in this case) or possibly the ethylene-containing backbone of PEI-6 is responsible for a certain degree of non-ionic Hb binding. The addition of less hydrophobic moieties decreases this interaction, while adding even more hydrophobic groups increases the overall effect as previously noted. Since all epoxide derivatives decreased total Hb-binding capacity (Hb_{iec} + Hb_{iso}) relative to the underivatized material,

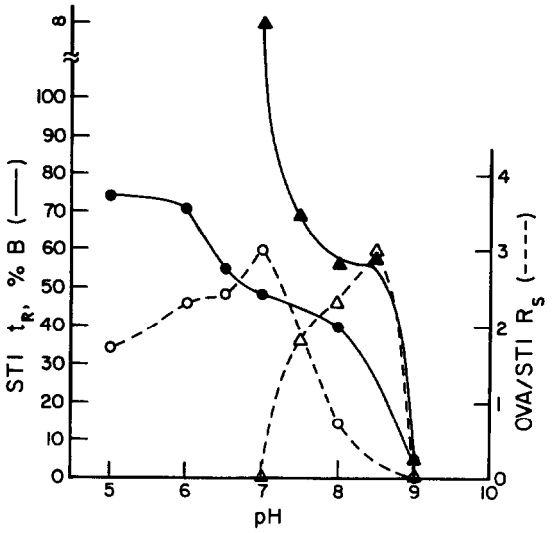


Fig. 5. Retention and resolution maps of probe proteins chromatographed on the GLY (●,○) and ENPP (▲,△) weak anion-exchange columns. The t_R (——) of STI and R_s (– – –) between OVA and STI were measured as a function of pH. (See Materials and methods; failure of STI to elute at 100% "B" is denoted by $t_R = \infty$.)

steric factors may play a greater role in protein binding (*i.e.* loading capacity) than in binding affinity.

Retention and resolution maps

Retention and resolution maps were constructed for all of the previously discussed stationary phases. Representative plots for STI t_R and OVA–STI R_s on the GLY- and ENPP-derivatized materials are illustrated in Fig. 5. (An analogous "map" for STI on the underivatized PEI-coated support falls between those from the GLY and ENPP stationary phases. In the interest of clarity, this data was not included in Fig. 5.) Examination of the retention curves shows that t_R generally decreased with increasing eluent pH. However, STI t_R was noticeably more pH-sensitive on the ENPP than on the GLY derivative. This implies that surface characteristics other than electrostatic charge contribute to the retention process. A comparison of the t_R curves in Fig. 5 to STI t_R on the stong anion-exchange support (Fig. 2) shows striking differences. Since the quaternized amine version of this support material possesses the maximum amount of surface charge theoretically possible, increases in STI t_R beyond that observed for the quaternary column were interpreted as being related to either (1) increased charge density, (2) a dual ionic–hydrophobic retention mechanism, (3) a decrease in the dielectric constant of the medium surrounding ionic sites on the support, or (4) some combination of the above. Unfortunately, it was not possible to distinguish between these phenomena under stated experimental conditions. Decreased retention compared to that on the strong anion-exchange support was viewed to result from the inverse of the aforementioned.

The R_s between OVA and STI chromatographed on the GLY and ENPP derivatives was also pH-dependent. In general, R_s increased with t_R until band-spread-

ing overwhelmed gains in selectivity. Although the ENPP derivative provided a R_s value of 3.0 at pH 8.5, the magnitude of pH sensitivity was such that this column could only be operated over a 1-pH-unit range (7.5–8.5). In contrast, the GLY derivative reached maximum R_s at pH 7.0 and was operable over a 3.5-pH-unit range. The fact that each column required a different operating pH to achieve maximum R_s testifies to the complex relationship between eluent pH, support surface charge and hydrophobicity. Thus, it appears difficult to anticipate chromatographic performance of a weak anion-exchange column without some knowledge of the chemical and physical characteristics of the support material.

Crosslinking reagents

Earlier data suggested that there was some non-ionic Hb binding associated with the underivatized crosslinked PEI matrix, and that subsequent amine derivatization could either increase or decrease its magnitude. To investigate the possibility that the crosslinker itself may be responsible for non-ionic Hb binding, additional crosslinking reagents were evaluated. (Researchers[6,22] have previously noted changes in the chromatographic performance of PEI-coated supports when crosslinked with different moieties.) Four multifunctional epoxides, whose structures (Table I) suggest some hydrophobic diversity, were examined: 1,2-ethanediol diglycidyl ether (EDGE); 1,4-butanediol diglycidyl ether (BUDGE); 1,3-diglycidylglycerol (DGG); and triglycidylglycerol (TGG). Using the OH:C ratio of the ring-opened structure to provide a rough estimation of hydrophobicity, these crosslinking reagents would be ranked in the following order: DGG < EDGE, TGG < BUDGE. Although evaluation data were not entirely as anticipated, a range of binding and chromatographic behavior was observed (Table V).

Different IPC values were obtained on each of these materials. This could have resulted from variations in reagent purity and/or reactivity. However, the ligand densities were sufficiently close for qualitative comparisons. The BUDGE- and EDGE-crosslinked surfaces were very similar, with BUDGE exhibiting only slightly more non-ionic Hb binding. TGG appeared to be more hydrophobic than BUDGE, which is in disagreement with the predicted rankings. Since elemental analysis of these materials showed that, for both TGG and BUDGE, 7–9 molecules of epoxide

TABLE V

EFFECT OF VARIOUS CROSSLINKING REAGENTS

*Crosslinking Reagent**	*IPC (μmol N/g support)*	*Hb_{iec}***	*Hb_{iso}***	*Hb_{tot}***	*iec/iso*	*t_R (% "B")****				*R_s§ (OVA–STI)*
						MYO	*OVA*	*STI*	*FER*	
EDGE	336	18	20	38	0.9	3	39	47	72	2.1
BUDGE	311	17	22	39	0.8	3	49	61	91	2.2
DGG	362	30	16	46	1.9	3	44	52	76	1.4
TGG	356	19	26	45	0.7	6	48	71	NE	2.6

* See Table I for structures.

** All values are mg Hb/g of support material; Hb_{tot} = total Hb-binding capacity.

*** Same chromatographic conditions as in Table II.

§ R_s calculated by the equation given in Materials and methods (*Chromatographic evaluation* section).

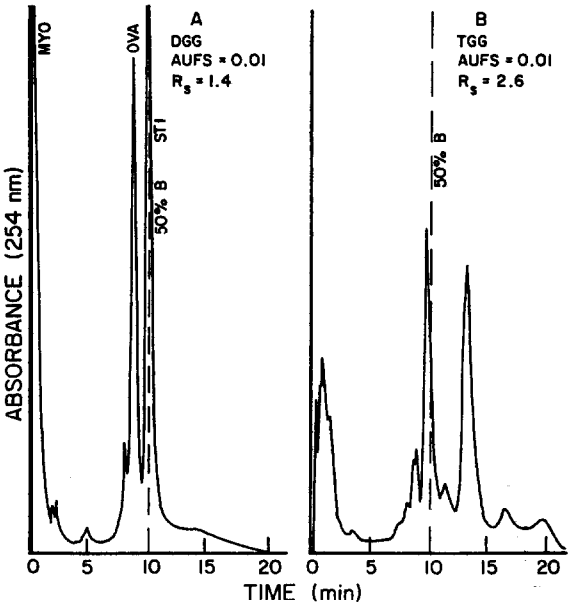


Fig. 6. The effect of crosslinking reagent hydrophobicity on retention and resolution. A three-component protein mixture (MYO–OVA–STI) was chromatographed on underivatized supports crosslinked with different multifunctional epoxides (DGG *vs.* TGG). Chromatographic conditions same as for Fig. 3.

reacted per PEI-6 molecule, one can assume that either the glycerol etherate backbone or some impurity in TGG imparts more hydrophobic character than expected.

A more illustrative comparison can be made between diglycidyl- and triglycidylglycerol. Both chromatographic (Fig. 6) and Hb-binding (Table V) data showed the TGG-crosslinked support to be more hydrophobic than the DGG-crosslinked material. In fact, FER could not be desorbed from the TGG column but could be eluted from the DGG column. The DGG-crosslinked support performed similarly to the EB-derivatized material (crosslinked with EDGE) discussed earlier. The use of DGG for crosslinking produced a more desirable stationary phase for the chromatography of high-molecular-weight proteins than the TGG-crosslinked matrix.

CONCLUSIONS

It was possible to manipulate protein retention and resolution on HPAEC columns through chemical modification of the stationary phase. Support materials synthesized with different ligand densities, amine substituents and crosslinking reagents exhibited unique chromatographic properties.

Although OVA–STI R_s increased with increasing ligand density, the recovery of FER decreased. Decreasing surface ligand density improved FER desorption at the expense of OVA–STI R_s. Apparently, a single HPAEC packing material cannot perform equally well with all proteins; therefore, it may be necessary to develop coatings of different ligand densities for optimum use with different classes of proteins.

Once a PEI-coated surface of given ligand density is obtained, retention and resolution can be modified by derivatizing surface amines with electrophilic reagents such as monofunctional epoxides. Amine derivatization with relatively hydrophobic epoxides, *e.g.* ETP or EPP, produced stationary phases with relatively high Hb_{iso} (low iec/iso ratios), t_R and R_s values; however, FER could not be desorbed. In contrast, the same coated silica derivatized with a less hydrophobic epoxide, *e.g.* EP, showed a relative decrease in Hb_{iso}, (increased iec/iso) t_R and R_s and allowed FER to be eluted. All epoxide derivatizations lowered total Hb-binding capacity compared to underivatized coated silica, possibly as a result of steric inhibition of Hb binding.

Hofstee and co-workers[14,15] have suggested that the mechanism underlying such chromatographic behavior involves both ionic and hydrophobic interactions. The protein may initially be captured electrostatically but, once brought into close proximity to the stationary phase, a secondary hydrophobic interaction could occur. Thus, the strength of the protein-support interaction would be the sum of both ionic and hydrophobic contributions. It was also suggested that purely electrostatic phenomena could be responsible. Instead of a secondary hydrophobic interaction, the magnitude of the ionic interaction itself may be altered by modification of surface amines. The addition of polar moieties would be expected to increase the dielectric constant associated with the support surface thereby decreasing the strength of the interaction[23], while non-polar substituents should have the opposite effect. In reality, retention is probably the result of both mechanisms.

Mobile phase pH had a significant effect on chromatographic retention and resolution. Retention mapping showed an inverse relationship between resolution and retention on all the weak anion-exchange stationary phases. Polyamine stationary phases derivatized with relatively hydrophobic epoxides were more sensitive to eluent pH than those with hydrophilic substituents.

Chromatographically, this was manifested by a fairly narrow pH-operating range. Two proteins, OVA and STI, of "constant" charge were used to "map" resolution on these stationary phases. Resulting plots showed that each support required a different eluent pH to achieve maximum resolution. For example, maximum OVA–STI R_s on the GLY and ENPP derivatives was at pH 7.0 and 8.5, respectively. These unique maxima may result from differential binding affinity and/or kinetic phenomena. The ionic and hydrophobic diversity of macromolecules suggests that the retention and resolution maps of other proteins may be quite different. Therefore, it is obviously not possible to predict chromatographic behavior without some knowledge of both stationary phase composition and protein character.

The evaluation of PEI-coated stationary phases interconnected with different multifunctional epoxides showed that the crosslinking reagent itself was somewhat responsible for non-ionic Hb binding. A coating crosslinked with DGG was chromatographically similar to the EB-derivatized coating. The use of TGG for crosslinking produced a relatively hydrophobic surface.

The optimal surface charge characteristics for an HPAEC stationary phase will depend on protein physical character. Small weakly-charged proteins may require a high ligand density surface to attain reasonable retention and resolution. Conversely, recovery of large, tenaciously-retained proteins will be expedited by the use of lower ligand density supports. In contrast, the utility of stationary phases with mixed ionic–hydrophobic character for protein separations may not be as straightforward.

Uniquely selective matrices can be obtained. However, non-ionic binding of some proteins to the more hydrophobic supports may poison the chromatographic surface, decreasing its loading capacity on subsequent runs (unpublished data). This will most likely not be a problem with analytical separations, since small sample loads and column cleaning procedures are commonly used. The applicability of these stationary phase materials to preparative chromatography will depend on the degree of non-ionic binding exhibited by proteins in the crude mixture. Future work will investigate the generality of the non-ionic binding phenomenon in addition to examining milder eluents for desorption.

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