Journal of Chromatography, 499 (1990) 177–196
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1672

HYDROPHILIC-INTERACTION CHROMATOGRAPHY FOR THE SEPARATION OF PEPTIDES, NUCLEIC ACIDS AND OTHER POLAR COMPOUNDS

ANDREW J. ALPERT
**PolyLC, 9052 Bellwart Way, Columbia, MD 21045 and Department of Medicine, The Johns Hopkins School of Medicine, Baltimore, MD 21205 (U.S.A.)*

SUMMARY

When a hydrophilic chromatography column is eluted with a hydrophobic (mostly organic) mobile phase, retention increases with hydrophilicity of solutes. The term hydrophilic-interaction chromatography is proposed for this variant of normal-phase chromatography. This mode of chromatography is of general utility. Mixtures of proteins, peptides, amino acids, oligonucleotides, and carbohydrates are all resolved, with selectivity complementary to those of other modes. Typically, the order of elution is the opposite of that obtained with reversed-phase chromatography. A hydrophilic, neutral packing was developed for use in high-performance hydrophilic-interaction chromatography. Hydrophilic-interaction chromatography is particularly promising for such troublesome solutes as histones, membrane proteins, and phosphorylated amino acids and peptides.

Hydrophilic-interaction chromatography fractionations resemble those obtained through partitioning mechanisms. The chromatography of DNA, in particular, resembles the partitioning observed with aqueous two-phase systems based on polyethylene glycol and dextran solutions.

INTRODUCTION

A hydrophilic, strong-cation-exchange (SCX) material, poly(2-sulfoethyl aspartamide)-silica (PolySulfoethyl A) was introduced recently[1] for high-performance liquid chromatography (HPLC) of peptides. The material is a complement or alternative to reversed-phase chromatography (RPC). Columns of this material are eluted with a salt gradient in a buffer at pH 2.8–3.0, often with some acetonitrile present. At low levels of CH_3CN, retention and selectivity are governed primarily by the number of basic residues or positive charges in peptides [1–3]. This property permits isolation and analysis of specific entities, such as C-terminal peptides[4], disulfide-linked peptides[5], and pyroglutamyl-terminated peptides[6]. The material is also useful, in combination with RPC, for peptide mapping[7] and purification of natural or synthetic peptides[1,5].

The selectivity changes markedly when the mobile phase contains high levels of organic solvent: (1) Retention increases dramatically above 70% CH_3CN, while elec-

0021-9673/90/$03.50

trostatic effects on the retention of Gly–Tyr diminish in importance[1]; (2) Hydrophilic peptides([Arg^8]-vasopressin, β-endorphin (1–16)) are retained more strongly relative to more hydrophobic peptides (somatostatin, β-endorphin (1–17))[1]; (3) A mixture of four decapeptide standards, differing only in hydrophobic character, is largely resolved with 50% CH_3CN[1], but not with lower concentrations[8]. The order of elution is least to most hydrophilic[8], opposite from the order in RPC.

These data suggest that increasing the proportion of organic solvent in the mobile phase increases the sensitivity of the sorbent to the hydrophilic residues of peptides. Retention due to such residues, normally a minor "mixed-mode" effect, seems to become a major factor above 70% CH_3CN. The same effect has been observed with a variety of stationary phases[9–11]. This is "normal-phase" chromatography in that elution is promoted by the use of more polar (here, more aqueous) mobile phases. However, a new term, hydrophilic-interaction chromatography, is proposed to describe the combination of hydrophilic stationary phases and hydrophobic, mostly organic mobile phases. A suitable acronym would be HILIC (HIC having been preempted by Hydrophobic-Interaction Chromatography). This is descriptive, as will be evident from the data to follow, and thus more accurate than the historical term "normal-phase chromatography".

The conditions of HILIC have been used extensively for the analysis of sugars and oligosaccharides[12–19] but only in isolated cases for other classes of compounds. The objectives of this report are: (1) to find out whether these disparate applications involve a single mode of chromatography; (2) to explore the utility of HILIC for the separation of polar solutes; (3) to introduce a packing designed for use in HILIC.

EXPERIMENTAL

HPLC apparatus and columns

Isocratic HPLC was performed with a Model 300 pump from Scientific Systems (State College, PA, U.S.A.), a spectroMonitor 3100 detector from LDC (Riviera Beach, FL, U.S.A.), and a Lloyd Instruments recorder, Model GRAPHIC 1000 (Vector Group, Newburgh, NY, U.S.A.). Gradient elution HPLC was performed with a 5500 liquid chromatograph, a VISTA 402 data system and a UV200 detector, all from Varian (Walnut Creek, CA, U.S.A.). The HPLC columns are manufactured by PolyLC (Columbia, MD, U.S.A.). PolySulfoethyl A and PolyHydroxyethyl A are available under the tradenames PolySulfoethyl Aspartamide and PolyHydroxyethyl Aspartamide, respectively. The C_{18} column was manufactured by Column Engineering (Ontario, CA, U.S.A.). The particle diameter was 5 μm unless noted otherwise; the pore diameter was 300 Å.

Titanium frits were obtained from Mott Metallurgical (Farmington, CT, U.S.A.). Ultra High Molecular Weight (UHMW) polyethylene frits were a gift from Upchurch Scientific (Oak Harbor, WA, U.S.A.). Alumina frits were purchased from Alltech (Deerfield, IL, U.S.A.).

Reagents

Cyclo(Ala–Ser), cyclo(Ala-Gly) and cyclo(Ser–Ser) were purchased from Research Plus (Bayonne, NJ, U.S.A.); [Des-Gln]-Substance P from Chemical Dynamics (S. Plainfield, NJ, U.S.A.); oxytocin, [Arg^8]-vasopressin, somatostatin, Substance

P(4–11) and Substance P(5–11) from Bachem (Torrance, CA, U.S.A.); Substance P(6–11) and Substance P(7–11) from Peninsula Laboratories (Belmont, CA, U.S.A.); and oligothymidylic acid $pd(T)_{25-30}$ from Pharmacia LKB (Piscataway, NJ, U.S.A.). Oligothymidylic acid $pd(T)_{12-18}$ and $pd(T)_{19-24}$ and all other peptides, amino acids, phosphorylated amino acids, organic acids, and oligonucleotides were obtained from Sigma (St. Louis, MO, U.S.A.).

The mixture of homologous 3-hydroxy-2-nitropyridinyl-β-D-maltooligoglycosides, as well as purified calibration standards, were a gift from Dr. A. M. Fathy (Diagno-Chemie, Stolberg, F.R.G.).

Organic reagents were purchased from Aldrich (Milwaukee, WI, U.S.A.). All reagents and solvents were of HPLC grade or of the purest grade available.

Preparation of mobile phases

Stock solutions of triethylamine phosphate (TEAP), 2 *M* in phosphate, were prepared by addition of triethylamine (TEA) to a concentrated solution of phosphoric acid till the desired pH was obtained, followed by appropriate dilution. This stock solution was stored in the refrigerator when not in use. The same method was used to prepare stock solutions of other salts not readily obtainable. Since the pH of amine salt buffers tends to drift, stock solutions should be prepared fresh monthly.

Mobile phases containing CH_3CN were prepared by addition of stock solution and the required volume of water to a volumetric flask, followed by addition of CH_3CN to a level several ml below the mark. After mixing, the flask was placed in a sonicator bath for 5 min, which both degasses and warms the cool solution. CH_3CN was then added to the mark.

Preparation of PolyHydroxyethyl A

PolyHydroxyethyl A was prepared by the incorporation of ethanolamine into a coating of polysuccinimide, covalently bonded to silica, using a procedure similar to that detailed in previous papers on polysuccinimide-based coatings[20,21].

RESULTS

Retention vs. hydrophilicity

Fig. 1 shows the separation of amino acids with a column of PolySulfoethyl A and a mostly organic mobile phase. The order of elution is approximately the opposite of that expected for RPC. This is quite evident in Fig. 2, which compares the relative order of elution of the non-basic amino acids (except for cysteine, which tailed badly) on several types of columns. PolySulfoethyl A and PolyHydroxyethyl A (described below) were used in the HILIC mode and displayed elution orders similar to each other and nearly the opposite of that reported for RPC[22]. Minor changes in the order of elution were observed with changes in the pH and salts used in the HILIC mobile phases (*e.g.*, a reversal of the position of Gly and Ser). The elution order is also given for a sulfonated polystyrene–divinylbenzene (PS–DVB) SCX packing, typically used in amino acid analyzers[23]. The elution orders in Fig. 2 suggest that separation on this column is due to relative hydrophobic interaction.

The same effects are observed with non-electrolytes, albeit at higher concentrations of CH_3CN. The cyclopeptides listed in Fig. 3 lack ionizable groups, the N- and

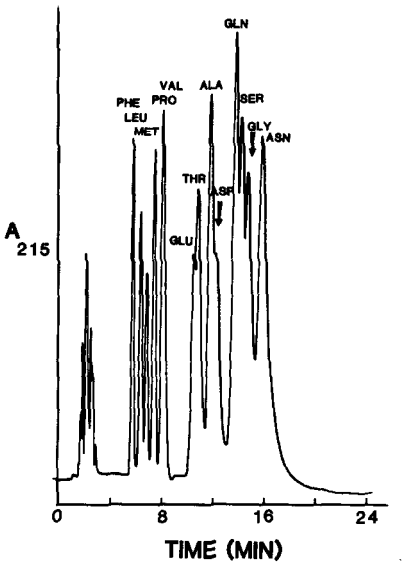

Fig. 1. HILIC of amino acids. Column, PolySulfoethyl A, 200 × 4.6 mm; mobile phase (isocratic), 5 m*M* TEAP (pH 2.8) with 80% CH_3CN; flow-rate, 2 ml/min.

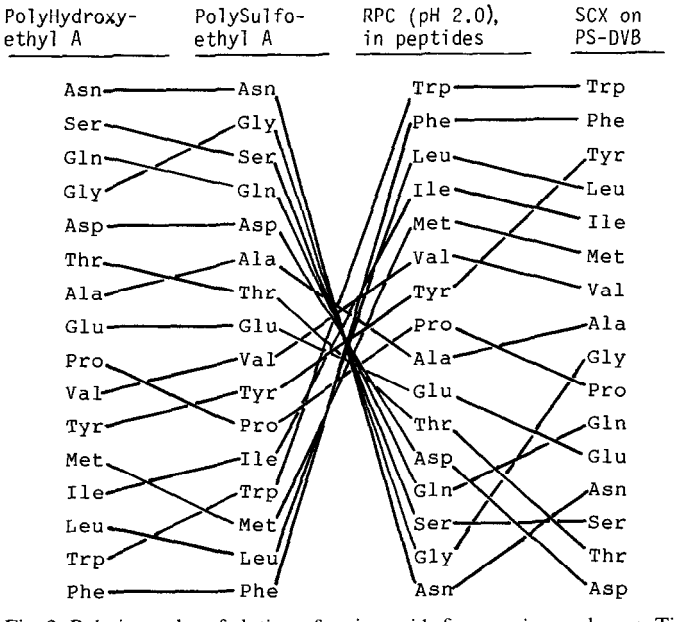

Fig. 2. Relative order of elution of amino acids from various columns. Tie lines connect the same amino acid. PolyHydroxyethyl A and PolySulfoethyl A: columns, 200 × 4.6 mm; mobile phase (isocratic), 10 m*M* TEAP (pH 2.8) containing 80% (v/v) CH_3CN. RPC: from ref. 22; Linear AB gradient from water to CH_3CN (0.1% TFA in each). SCX: from ref. 23; Step gradient of increasing salt and pH.

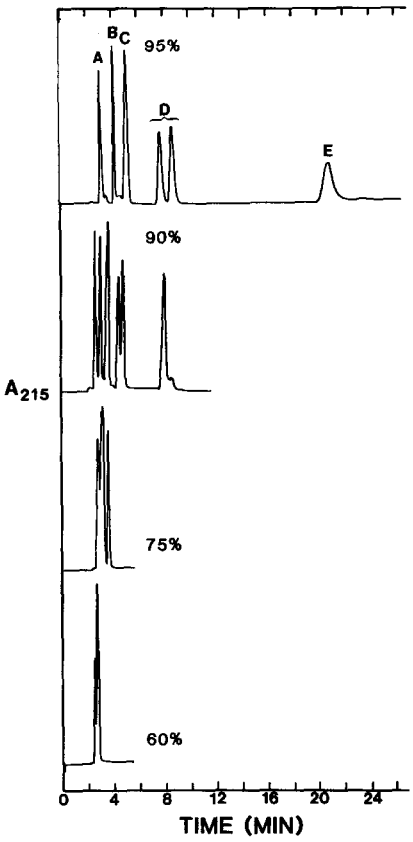

Fig. 3. HILIC of cyclopeptides. Column, as in Fig. 2; mobile phase (isocratic), 15 m*M* TEAP (pH 2.8) with 95, 90, 75 or 60% CH_3CN as noted; flow-rate, 1 ml/min. Peaks: A = cyclo(Leu–Gly); B = cyclo(Phe–Ser); C = cyclo(Ala–Gly); D = cyclo(Ala–Ser); E = cyclo(Ser–Ser).

C-termini being linked in an amide bond to form a diketopiperazine ring. Nonetheless, retention increases with hydrophilicity of the residues involved and is inversely proportional to the polarity of the mobile phase.

All these cyclopeptides were eluted in doublets depending on the mobile phase used, as is evident here for cyclo(Ala–Ser). Presumably these correspond to geometric isomers, since the corresponding linear peptides, with blocked termini, gave single peaks (data not shown).

Preparation of poly(2-hydroxyethyl aspartamide)-silica

To facilitate the study of the HILIC mode, a new HPLC packing was synthesized (Fig. 4). This material is poly(2-hydroxyethyl aspartamide)-silica, or Poly-Hydroxyethyl A. The new material, more hydrophilic than PolySulfoethyl A (see below) but lacking its charged character, is well suited for the study of HILIC.

HILIC of amino acids on SCX and neutral supports

The graphs in Fig. 5 show HILIC effects on retention of ordinary and

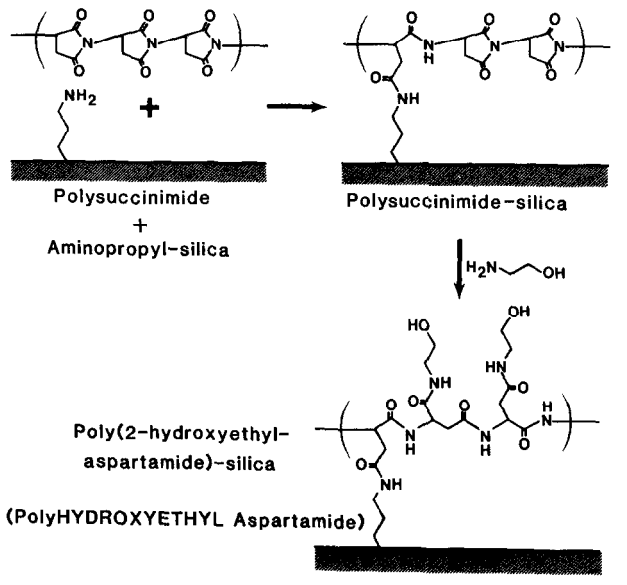

Fig. 4. Preparation of PolyHydroxyethyl A.

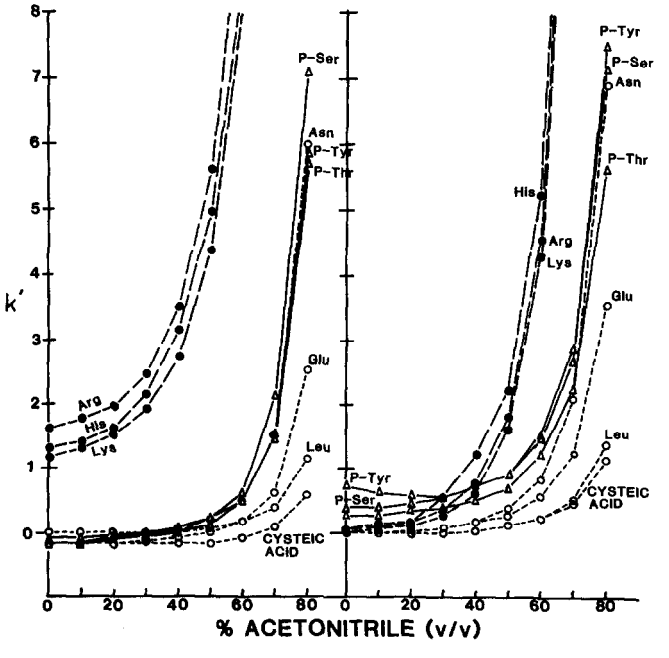

Fig. 5. HILIC (isocratic) of amino acids. The capacity factor was determined from the formula $k' = (t_R - t_0)/t_0$, where t_0 is the elution time for toluene, a non-retained solute, at a given flow-rate. Columns, 200 × 4.6 mm, with titanium frits; mobile phase, 25 m*M* TEAP (pH 5.0) with CH_3CN as noted. Left: Poly-Sulfoethyl A; right: PolyHydroxyethyl A.

phosphorylated amino acids. In the absence of an organic solvent, Arg- and His- are retained on PolyHydroxyethyl A (Fig. 5, right) to an insignificant extent. All other ordinary amino acids are eluted in the void volume. There is a dramatic increase in retention of the basic amino acids as the organic solvent content of the mobile phase is increased. This suggests that positively charged amino acids are quite hydrophilic. The same is true of negatively charged amino acids, as evidenced by the pronounced retention of phosphorylated amino acids in the HILIC mode; for example, phosphorylation increases the capacity factor (k') of Tyr- by one order of magnitude. However, not all charged species are equally hydrophilic; k' increases markedly for the series cysteic acid < glutamic acid < phosphorylated amino acids. In fact, cysteic acid is less well retained than is Leu-. Neutral amino acids are well retained and resolved, starting at *ca.* 70% CH_3CN.

HILIC effects on PolySulfoethyl A (Fig. 5, left) are similar, but are superimposed on electrostatic effects. Even in the absence of organic solvents, the basic amino acids are well retained. Values for k' increase with organic solvent concentration, probably due to hydrophilic interactions, since the shape of the retention curves are the same in both graphs. Above 50% CH_3CN, hydrophilic interactions are more prominent than electrostatic effects, which contribute to retention as a minor "mixed-mode" component.

The negatively charged Glu-, cysteic acid, and phosphorylated amino acids are eluted from PolySulfoethyl A before the void volume ($k' = -0.15$) in the absence of or at low levels of organic solvent, indicating electrostatic repulsion. However, at CH_3CN levels above 50%, HILIC effects overcome this repulsion, and thereafter, retention is similar on both columns.

Phosphorylated amino acids are retained and resolved on PolyHydroxyethyl A, even in the absence of organic solvents (Figs. 5 and 6). This makes their analysis in protein hydrolyzates convenient, since other amino acids are not retained under these conditions (Fig. 6), although the separation of P-Thr- and P-Ser- needs to be optimized. The order of elution does not correlate with that of the corresponding non-phosphorylated amino acids (Fig. 2). P-Tyr- is anomalously well retained. This is also

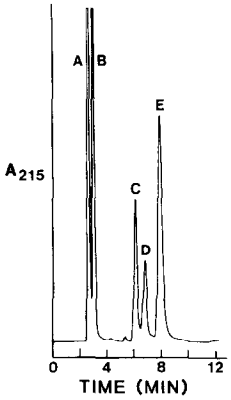

Fig. 6. HILIC of phosphorylated and non-phosphorylated amino acids. Column, PolyHydroxyethyl A (3 μm), 100 × 4.6 mm, with titanium frits; mobile phase (isocratic), 10 m*M* TEAP (pH 2.8); flow-rate, 0.5 ml/min. Peaks: A = His-; B = Ser-, Leu- (void volume); C = P-Thr-; D = P-Ser-; E = P-Tyr-.

the order of elution observed with anion-exchange columns[24,25]. This suggests that the PolyHydroxyethyl A column may be operating as a low-capacity anion-exchange column, a view supported by the elution of basic amino acids in doublets with mobile phases of low ionic concentration. One peak of the doublets coincides with and the other precedes the void volume, suggesting an element of electrostatic repulsion. This would be readily apparent in Fig. 6, for example, except that the presence of Ser- and Leu- in the void volume masks the void volume peak of the His- doublet. However, raising the concentration of the mobile phase from 10 to 25 m*M* (Fig. 5) not only eliminates the peak splitting, but leads to negligible retention of Arg- and His- (k' = 0.1). Thus, the interpretation of Fig. 6 may not be a straightforward matter. In any case, the selective retention of phosphorylated amino acids raises the possibility of the selective isolation of phosphorylated peptides from a protein digest.

In the course of this study, it was observed that phosphorylated amino acids did not pass through HPLC columns with stainless-steel frits from certain manufacturers. The problem was not encountered with frits of titanium, alumina, or UHMW polyethylene. It was subsequently learned[26] that the recovery of phosphorylated peptides

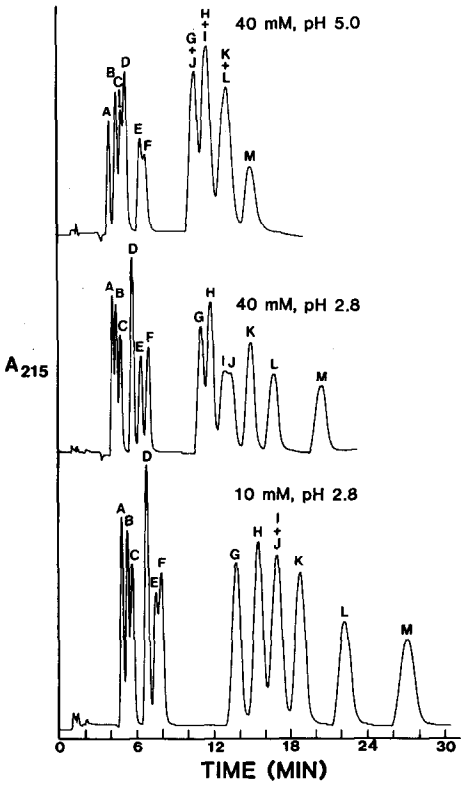

Fig. 7. HILIC of dipeptide standards. Column, PolyHydroxyethyl A, 200 x 4.6 mm; mobile phase (isocratic), TEAP buffer (concentration and pH as noted) with 80% CH_3CN; flow-rate, 2 ml/min. Peaks: A = Gly–Phe; B = Gly-Leu; C = Gly–Ile; D = Gly–Met; E = Gly–Val; F = Gly–Tyr; G = Gly–Ala; H = Gly–Glu; I = Gly–Asp; J = Gly–Thr; K = Gly–Gly; L = Gly–Ser; M = Gly–Asn.

from HPLC columns improved significantly when stainless-steel frits were replaced by titanium frits. These data imply that non-ferrous frits should be used in HPLC of phosphorylated species.

HILIC of peptides

Fig. 7 shows the separation, under HILIC conditions, of dipeptides with the composition Gly-X on a column of PolyHydroxyethyl A. As with the amino acids, retention appears to be governed by hydrophilic interactions, increasing with polarity of the solute. Retention is inversely proportional to pH and ionic concentration.

Fig. 8 (I) shows the resolution by HILIC of peptide standards with varied composition. The coincidence of Tyr–Gly and Gly–Tyr is an example of the insensitivity of the HILIC mode to a change of sequence that does not affect charge (and thus hydrophilicity). By contrast, location of the acidic residue of Asp–Val next to the N-terminus probably creates an ion pair in which the charged centers are close enough to neutralize each other. Evidently this decreases their hydrophilicity, in view of the greater retention of Val-Asp, where the charged centers are more isolated. Glu–Ala-Glu provides a good demonstration of the sensitivity of HILIC to the composition of the hydrophilic residues in a peptide, since addition of an Asn- residue nearly doubles the k' of the peptide. This addition also moves the C-terminal carboxyl group further away from the carboxyl of the side chain of Glu-. However, the Asn-residue probably does not promote retention through electrostatic effects, since a similar increase is observed with the addition of Gln- residues to the N-terminus of small peptides with neutral N-terminal residues (see Fig. 9).

Chromatogram II of Fig. 8 shows the (non-optimized) separation by HILIC of

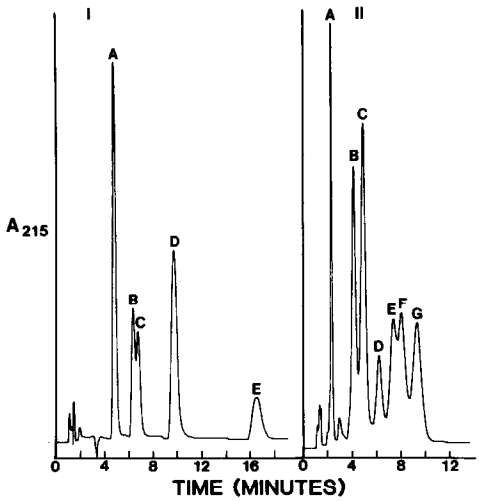

Fig. 8. HILIC of peptide standards (isocratic). Column, as in Fig. 7; mobile phase, 40 m*M* TEAP (pH 2.8) with 80% (I) or 70% (II) CH_3CN; flow-rate, 2 ml/min. Peaks: (I) A = Asp–Val; B = Val–Asp; C = Tyr–Gly + Gly–Tyr; D = Glu–Ala–Glu; E = Glu–Ala–Glu–Asn; (II) A = Oxytocin; B = [Tyr^1]-somatostatin; C = somatostatin; D = [Tyr^{11}]-somatostatin; E = [Des-Gln]-Substance P; F = Substance P; G = [Arg^8]-vasopressin.

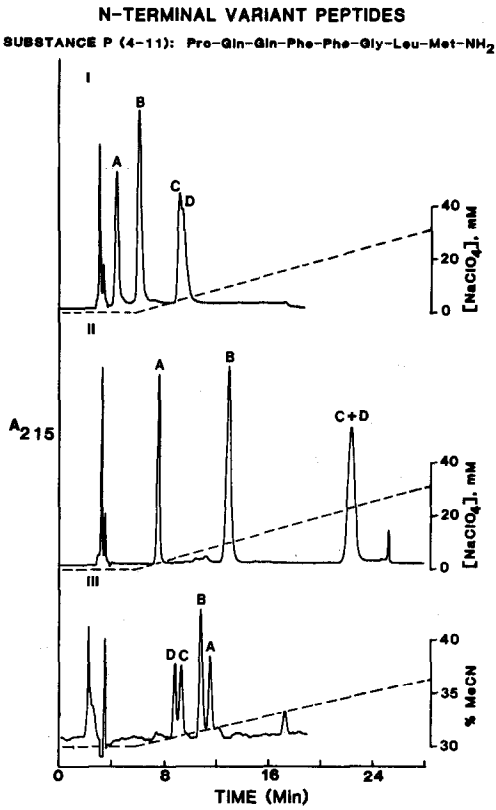

Fig. 9. Gradient elution of Substance P fragments from (I) a PolyHydroxyethyl A column (HILIC mode); (II) a PolySulfoethyl A column (HILIC mode); (III) a C_{18} column (RPC mode). Column dimensions, 200 × 4.6 mm; flow-rate, 0.8 ml/min; detection, absorbance at 215 nm, 0.5 a.u.f.s. Elution: (I), (II) 90-min linear gradient, 0–100% eluent B. Eluent A, 10 m*M* TEA-methylphosphonate (pH 3.0) with 80% CH_3CN. Eluent B, same + 125 m*M* sodium perchlorate. (III) 90-min linear gradient, 0–100% eluent B. Eluent A, 0.1% TFA in 30% CH_3CN; eluent B, 0.1% TFA in 55% CH_3CN. Peaks: Substance P fragments: A = (7–11); B = (6–11); C = (4–11); D = (5–11). MeCN = Acetonitrile.

some polypeptide hormones. The concentration of CH_3CN used was lower than for chromatogram I in order to achieve isocratic elution of these peptides in a reasonable time. With the exception of oxytocin, the order of elution is the opposite of that observed for RPC[27] and HIC[28]. As may be surmised from the amino acid data, basicity of a peptide promotes retention, but to a much lesser extent than would be the case with a PolySulfoethyl A column operated in the cation-exchange mode; the time for the isocratic elution of peptides of +1, +2 and +3 charge is considerably shorter. At the same time, the sensitivity of the hydrophilicity of the nonbasic residues results in a selectivity complementary to that of cation exchange. Thus, while [Arg[8]]-vasopressin has a charge of +2, it is eluted after somatostatin, Substance P and their analogues, all with a +3 charge but also with more hydrophobic character.

N-Terminal variant peptides

Eucaryotic proteins, manufactured by recombinant methods, are sometimes

found to vary in composition, particularly at the N-terminus. There may be an additional N-terminal Met- or N-formyl Met- residue; conversely, exoprotease activity may remove one or two residues from the end. Assessment of the purity of a recombinant protein involves digestion of the product and analysis of the N-terminal peptide fraction for the presence of such variant sequences. RPC often fails to distinguish between such variants.

Fig. 9 shows the separation of a series of Substance P fragments with residues removed in sequence at the N-terminus. HILIC was used to obtain chromatograms I and II, while chromatogram III is RPC. Gradient rates were chosen to give comparable peak widths in both modes. While RPC features the best overall selectivity in this case, the degree of separation of these variant peptides is greatest with HILIC. This is particularly the case when the capacity is increased by the superposition of electrostatic effects and hydrophilic interactions, as with PolySulfoethyl A (chromatogram II). Selectivity differences with PolyHydroxyethyl A may reflect the "contact region" of the peptides (see Discussion).

Peptides may be eluted from HILIC columns with decreasing organic gradients as well as increasing salt gradients. This topic is addressed in the Discussion section, as is the selection of the salts.

Partition mechanisms and HILIC of carbohydrates

HPLC of carbohydrates has been performed since 1975[12–15,17,19] with columns of silica with an aminopropylsilane coating (NH_2-silica); mobile phases typically contain about 80% CH_3CN. These conditions are effectively those of HILIC. This implies that NH_2-silica retains carbohydrates not directly through its basic charge[15–17] but because the basic groups are hydrophilic. The uncharged, hydrophilic PolyHydroxyethyl A also works well for HILIC of carbohydrates. This is demonstrated in Fig. 10 with a homologous series of oligoglycosides and various concentrations of CH_3CN. Other hydrophilic stationary phases can also be used, such as diol-[15] and polyol-[16–18] type packings. PolySulfoethyl A can be used, but retention times are 25% less than with PolyHydroxyethyl A. Presumably, this reflects the relative hydrophilicity of these stationary phases.

Reducing sugars are well retained and resolved on PolyHydroxyethyl A, but are eluted in doublets corresponding to the α- and β-anomers (data not shown). This complicates the elution pattern. The problem is overcome by addition of a small amount of amine to the mobile phase; this speeds up the rate of mutarotation[15–17] and collapses the doublets.

Chromatography of carbohydrates as conducted above has been shown to involve a partitioning mechanism[29–31]. The stationary phase retains a semi-immobilized layer of mobile phase enriched with water. Chromatography of carbohydrates involves partitioning between this stagnant aqueous layer and the bulk of the (mostly organic) mobile phase. It would be reasonable to suppose that the partition mechanism advanced for the chromatography of carbohydrates represents the mechanism of HILIC fractionations of other classes of polar solutes as well.

Aqueous two-phase systems and HILIC of oligonucleotides

In order to gain additional insight into the mechanism of HILIC, the chromatography of oligonucleotides was studied. Nucleic acids can be fractionated by parti-

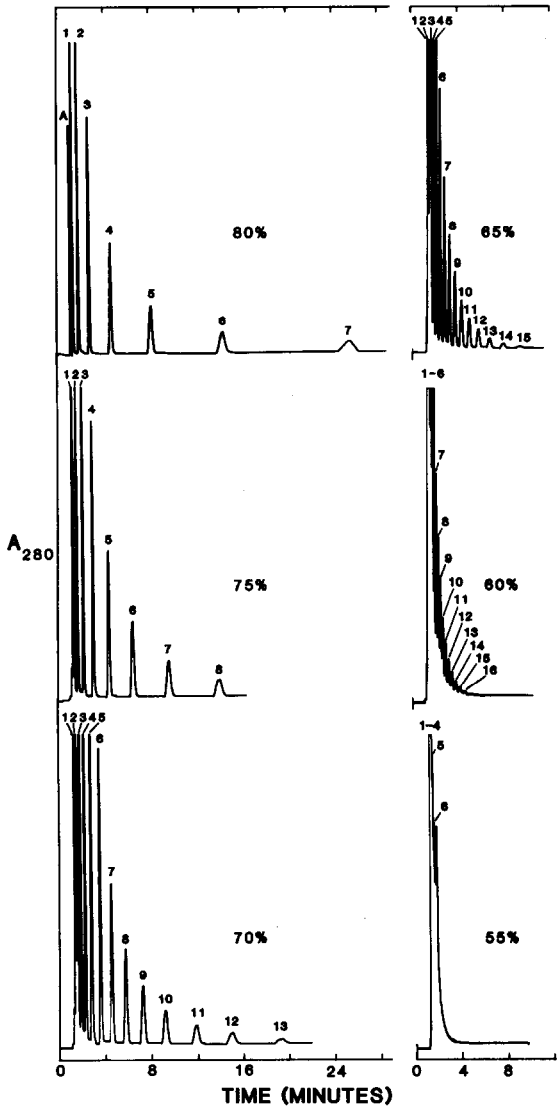

Fig. 10. HILIC (isocratic) of a homologous mixture of 3-hydroxy-2-nitropyridinyl-β-D-maltooligoglycosides. Peaks were identified with purified standards. Column, PolyHydroxyethyl A, 200 × 4.6 mm, 5 μm; mobile phase, acetonitrile–water, 80, 75, 70, 65, 60 or 55% acetonitrile as noted; flow-rate, 2 ml/min. Peaks: 1, 2, 3 ... = degree of polymerization; A = aglycon.

tioning between two aqueous phases, rendered immiscible by small amounts of two polymers that differ sufficiently in hydrophilicity, such as polyethylene glycol (PEG) and dextran. Albertsson[32] has developed the potential of such systems for the bulk fractionation of cells and proteins as well as nucleic acids, and Müller and Kutemeier[33,34] have applied the phenomenon to the chromatographic fractionation of nucleic acids.

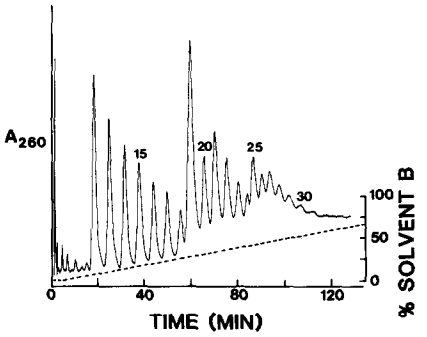

Fig. 11. HILIC (gradient elution) of oligothymidylic acid, $pd(T)_{12-30}$. Column, PolyHydroxyethyl A (3 μm), 100 × 4.6 mm; gradient, 200 min linear, 0–100% eluent B. Eluent A, 40 m*M* TEAP (pH 5.0) with 70% (v/v) CH_3CN. Eluent B, 75 m*M* TEAP (pH 5.0) with 80% (v/v) CH_3CN; flow-rate, 1 ml/min; detection, absorbance at 260 nm, 0.1 a.u.f.s.

Fig. 11 shows a homologous series of thymidylic acid [pd(T)] oligomers. A gradient of increasing salt was used; in addition, a slightly increasing gradient of CH_3CN concentration was employed to promote retention near the end of the salt gradient and thus increase resolution of closely related species. The results obtained with HILIC resemble those obtained with the above partitioning systems in the following respects: (1) Retention increases with oligomer size; (2) Retention of oligo-$pd(A)_6$ was about three times greater than that of oligo-$pd(T)_6$, while retention of oligo-$pd(C)_6$ and oligo-$pd(G)_6$ was more than one order of magnitude greater (the opposite of the trend observed with anion-exchange HPLC[35]); (3) Retention was extremely sensitive to the ionic strength of the mobile phase (permitting elution with unusually shallow salt gradients).

HILIC of organic acids and bases

A column of PolyHydroxyethyl A was eluted with 75 m*M* TEAP (pH 5.0), containing 70% CH_3CN. Glyceric acid, tartronic acid and phosphoglyceric acid were completely resolved, with k' values of 0.46, 1.29 and 1.75, respectively. This demonstrates once again the hydrophilicity of charged groups, especially phosphate groups.

Organic bases contain hydrophilic functional groups, and should lend themselves well to analysis by HILIC. In fact, HILIC conditions are already used for the analysis of basic drugs, both with uncoated silica[36,37] and NH_2-silica[38,39].

DISCUSSION

The mechanism of HILIC

The mechanism by which the HILIC mode operates can be discussed at several levels:

At the empirical level, HILIC is a variant of normal-phase chromatography. Retention is proportional to the polarity of the solute and inversely proportional to the polarity of the mobile phase.

The data in this paper suggest that the mechanism of HILIC involves partitioning between the (hydrophobic) mobile phase and a layer of mobile phase enriched

with water and partially immobilized on the stationary phase. This distinguishes this variant of normal-phase chromatography from other variants involving adsorption directly on the stationary phase. The following observations support a partitioning mechanism: (1) Analogy with the partitioning mechanism already worked out for the fractionation of carbohydrates; (2) The decreasing differences between charged and uncharged stationary phases with increasing levels of organic solvent in the mobile phases (*cf.* Fig. 5); (3) In HILIC of oligonucleotides, the sensitivity to factors known to affect the partitioning of nucleic acids between PEG-containing aqueous phases and (more hydrophilic) dextran-containing aqueous phases: ionic concentration, base composition, and molecular weight. In fact, 25% CH_3CN can substitute for the PEG in such two-phase systems[40].

The phenomena responsible for the partitioning of solutes between the two layers are obscure. Some form of dipole–dipole interactions may be involved, although Nikolov and Reilly[31] found that retention of sugars under HILIC conditions was better correlated with their hydration number than with the potential to form hydrogen bonds. The partitioning of solutes in aqueous two-phase systems is not well understood either[32]. A great deal of study will be required to clarify these questions.

HILIC as a mixed-mode effect in RPC

One can increase the retention of solutes through hydrophilic interactions by increasing the concentration of organic solvent in the mobile phase. For example, when Nikolov and Reilly[31] eluted a column of uncoated silica with 90% CH_3CN, they obtained k' values for sugars comparable to those obtained by elution of NH_2-silica with 80% CH_3CN. This raises the question of how hydrophobic a stationary phase can be and still be used in the HILIC mode if one is prepared to use levels of organic solvent approaching 100%. An extreme case of this may be RPC packings that are not end-capped. On such columns, the retention of many proteins and peptides as a function of % CH_3CN is a U-shaped curve[41–49]. Bij *et al.*[41] have ascribed retention at lower CH_3CN levels —the conventional RPC region— to "solvophobic" interactions and retention on the other side of the minimum to "silanophilic" interactions.

A reinterpretation of these observations may be warranted by the data in the present study. Retention in the organic solvent-rich region of the U-shaped curves may reflect hydrophilic interactions. In that case, the order of elution of solutes should be the reverse of that in the other branch of the curve. This is in fact the case for peptides[42,43], proteins[46–49], and small molecules[9]. Bij *et al.*[41] even obtained U-shaped curves for polar solutes by eluting silica in a reversed-phase mode. Their explanation is succinct: "...it simply demonstrates that the polarity of the eluent relative to that of the stationary phase determines whether the retention behavior is 'normal' or 'reversed'." This explanation will serve as well, at the empirical level, for the results presented here.

Cyclodextrin-bonded phases offer vivid examples of these effects. With mobile phases low in organic solvent, retention is of the RPC type, through inclusion-complex formation with the hydrophobic cyclodextrin cavity[50]. Retention at high levels of organic solvent is evidently due to HILIC, reflecting the hydrophilic nature of the exterior of the cyclodextrins. Thus, plots of k' *vs.* % CH_3CN are U-shaped curves for dansylated amino acids[51], showing at least partial inversion of the elution order.

These stationary phases also resolve carbohydrates satisfactorily at high CH_3CN levels[52,53], again reflecting a possible HILIC mechanism.

RPC is probably a "mixed-mode" process, reflecting the presence of both hydrophobic and hydrophilic interactions. The hydrophobic interactions are negligible at high levels of organic solvent. The data from this study suggest that the hydrophilic interactions are negligible at low levels of organic solvent, except for the most hydrophilic solutes observed here: organic bases. Such compounds are notorious for tailing in RPC. The phenomenon is usually ascribed to electrostatic attraction to silanols. A gradient of increasing organic solvent decreases retention through hydrophobic interactions, but would increase hydrophilic interactions. The presence of the latter as a mixed-mode effect would lead to the opposite of the focussing effect normally expected from gradient elution and cause tailing. It should be noted that any mobile phase component that decreases retention in HILIC also decreases tailing of basic compounds in RPC. Agents used for this purpose include amines[41], amine salts[41] and inorganic salts[27]. Hydrophilic amines are less effective for this purpose than hydrophobic amines[41], an observation difficult to explain in terms of electrostatic effects but consistent with HILIC effects. It may also be noted that U-shaped curves are not obtained if no hydrophilic interactions are possible, whether due to a lack of polar sites in the stationary phase[9,54] or to the non-polar nature of a particular solute[41].

The contact region of peptides

Some HILIC effects can be understood in terms of the "contact region" concept developed to describe the region of a protein's surface that preferentially makes contact with a chromatographic sorbent[55]. For example, Substance P has basic (and thus hydrophilic) and hydrophobic domains at opposite ends of the molecule. This could permit the selective adsorption of the hydrophilic domain on a HILIC stationary phase surface. No such segregation of domains is obtained with somatostatin; a hydrophobic region is bracketed by two basic residues. This may explain the lesser retention of somatostatin compared to Substance P on both PolySulfoethyl A[1] and PolyHydroxyethyl A.

The same concept can account for the relatively poor ability of RPC to resolve N-terminal variant peptides and the success noted with HILIC (Fig. 9). As with other basic groups, a charged N-terminus is quite hydrophilic. In RPC, this will tend to exclude the N-terminus as a preferred contact region. An example of this was observed[28] in RPC of several nonapeptides with the same composition but different sequences. With a blocked N-terminus, retention was greater with three Leu- residues at this terminus than when they were located in the interior, presumably reflecting steric accessibility. The elution positions were reversed when the N-termini were unmodified.

The best mode for the analysis of N-terminal variant peptides must be determined on a case-by-case basis. The modes of greatest utility may be those in which the N-terminus is a preferred contact region. Cation-exchange certainly qualifies, as does a combination of cation-exchange and hydrophilic interaction (Fig. 9, chromatogram II). However, the electrostatic effects may be so marked as to lead to some insensitivity to the composition of the non-basic residues relative to the basic residues and the N-terminus itself. Thus, PolyHydroxyethyl A, operating purely through hydrophilic interaction, is better able than PolySulfoethyl A to discriminate between some peptides differing in the composition of non-basic residues (Fig. 9).

HILIC on miscellaneous stationary phases

A number of reports in the literature describe the use of hydrophilic stationary phases under HILIC conditions. Mant *et al.*[10] studied the use of SynChropak GPC for size separation of peptides. This neutral, reasonably hydrophilic packing displayed HILIC effects when the mobile phase contained over 60% CH_3CN (Fig. 6 in ref. 10). A similar packing, LiChrosorb DIOL, has also been used for HILIC (see below).

Carunchio *et al.*[11] coated LiChrosorb with chitosan. The resulting material was a hydrophilic, low-capacity anion exchanger. Nucleotides, amino acids and dipeptides were resolved under HILIC conditions; a mixture of hydrophilic interactions and electrostatic effects was evident. For example, electrostatic repulsion appears to have counteracted the pronounced hydrophilic interaction of the basic amino acids, leading to their elution in the same period of time as the other amino acids.

HILIC of proteins

Perhaps the first purifications of proteins with HILIC conditions were those of Rubenstein *et al.*[56,57]. Interferon[56] and serum proteins[57] were purified on LiChrosorb DIOL, using a decreasing gradient of 1-propanol.

Soluble proteins tend to become denatured in the presence of CH_3CN concentrations above 20%. Thus, HILIC would not be useful for the purification of most soluble enzymes. However, for many proteins a high organic solvent content in the eluent is of no concern. For example, some enzymes and proteins function in the hydrophobic milieu of membranes, and might be expected to respond well to chromatography with a mostly organic solvent mobile phase. Several reports have described the purification of integral membrane proteins by "ion-exchange" chromatography with such mobile phases; the sorbents used were the hydrophilic packings CM-Trisacryl[58–60] and DEAE-cellulose[61]. Yields and purity were appreciably higher than with the standard aqueous mobile phases containing surfactants.

With some protein mixtures, the objective may be simply to determine the number of components present. In the case of H1 histone from chicken erythrocytes[62], RPC yields 3 peaks, while HILIC on the cation exchanger PolyCAT A[20] yields 21 (using an increasing gradient of sodium perchlorate).

Practical aspects of HILIC mobile phases and gradients

Gradient elution in HILIC can be accomplished by increasing the polarity of the mobile phase, using either a decreasing organic solvent or an increasing salt gradient. When ion-exchange columns are used in the HILIC mode for a sample of unknown characteristics, then a salt gradient is preferable, since it insures the elution of most solutes in a reasonable time. By contrast, uncharacterized solutes are probably best eluted from PolyHydroxyethyl A columns with gradients of decreasing organic solvent [63]. This guarantees elution of all components in the sample, since they are retained by PolyHydroxyethyl A only through hydrophilic interactions. Salt gradients may then be evaluated for complementary selectivity. CH_3CN is a convenient solvent to use, since it yields less viscous mobile phases and greater retention of solutes than mobile phases containing equivalent amounts of methanol or 2-propanol. At levels of CH_3CN above 70%, every 1% increase leads to an increase of about 10% in the (isocratic) k' values for small molecules.

One of the problems with HILIC is the poor solubility of many salts in mobile phases containing high concentrations of organic solvents. Sodium perchlorate is one salt that is quite soluble in such media and which also does not absorb light at the low wavelengths (*ca.* 215 nm) frequently used for detection of peptides. Organic salts also offer reasonable solubility. Salts of phosphonic acids, such as sodium methylphosphonate, are promising. The buffering ranges and optical transparency of these salts are similar to those of the corresponding phosphate salts, and the solubility in HILIC mobile phases is superior. The same is true of amine salts such as TEAP. Unfortunately, TEA salts sometimes yield rising baselines and artifactual peaks in gradient elutions; this reflects the accumulation of oxidation products of TEA[64]. Sodium, potassium, and ammonium salts do not possess this liability. However, substitutions involving either counterion of a salt can affect the selectivity, sometimes in a dramatic fashion. This topic will be addressed in a future study.

Peptides can be purified for sequencing purposes using PolyHydroxyethyl A columns with volatile mobile phases, due to the low concentrations of salt required with this column. In such cases, a descending gradient of CH_3CN is convenient. A suitable salt is the triethylamine salt of trifluoroacetic acid[64]. This salt is both volatile and transparent at 215 nm. The TEA must be scrupulously purified to avoid the baseline artifacts described above. An alternative volatile salt is ammonium formate[65], although this is less volatile than the triethylamine salt of trifluoroacetic acid, and absorbs below 240 nm.

It is not necessary to include salts in the mobile phase for HILIC of nonelectrolytes, such as carbohydrates. However, omission of salt leads to broad peaks and variable retention times for amino acids and peptides.

The selectivity of HILIC complements that of RPC. The two modes, used in sequence, may afford useful fractionations of complex mixtures. It would be convenient to use HILIC as the second step, since a fraction collected from the RPC column would merely need to receive sufficient additional organic solvent to assure good binding to the HILIC column. The capacity of PolyHydroxyethyl A columns for peptides is several times less than that of suitable ion-exchange columns, and is comparable to that of an RPC column.

It may be difficult to elute polyelectrolytes from ion-exchange columns in the HILIC mode, owing to the high levels of salt required. An example would be the elution of very basic peptides from PolySulfoethyl A. In such cases, PolyHydroxyethyl A is a suitable alternative, requiring one-fifth to one-tenth as much salt. For the preparative purification of oligonucleotides, it is a particularly promising alternative to anion-exchange HPLC[35,66–70], which typically requires 0.5–2.0 *M* salt and often 5 *M* urea (which will interfere with sensitive biological experiments).

CONCLUSIONS

HILIC is potentially as general a mode as reversed-phase and ion-exchange chromatography. It affords good resolution within every class of polar solutes evaluated to date, and works best with hydrophilic solutes least suitable for fractionation by RPC. Thus, it has the potential to resolve mixtures not heretofore amenable to fractionation by chromatography. A variety of hydrophilic stationary phases can be used in the HILIC mode; the choice depends on the application. For example, an

ion-exchange column may display superior selectivity which reflects the superposition of electrostatic and HILIC effects, while PolyHydroxyethyl A may be preferred if a volatile mobile phase is necessary. Some properties of columns other than HILIC columns can also be ascribed to hydrophilic interaction, present as a mixed-mode effect.

Perhaps the most important single property described in this study is the pronounced hydrophilicity of charged, basic groups, which influences the chromatography of any solute to which they are attached. This hydrophilicity can account for the successful analysis of some mixtures by HILIC that are not successfully analyzed by RPC, as well as the tailing of basic compounds in RPC.

There are some applications for which HILIC seems to be especially well suited. PolyHydroxyethyl A shows a particular affinity for phosphorylated amino acids under some conditions, and may be useful for the selective isolation of phosphorylated peptides. Another is the analysis and purification of polyelectrolytes, such as very basic peptides and oligonucleotides, which are eluted from columns of PolyHydroxyethyl A at levels of salt far lower than those needed for ion-exchange columns. Purification of some membrane proteins and histones seems to be easier to achieve by HILIC than by other modes of chromatography.

PolyHydroxyethyl A is promising as a stationary phase for which HILIC effects are the only ones of significance. It is possible to prepare uncharged stationary phases that are still more hydrophilic. This would permit the operation of HILIC at lower levels of organic solvent. Such stationary phases are currently under development. The polyaspartamide coatings used in this work are stable polymers of derivatives of asparagine, the most hydrophilic neutral amino acid, and the packing materials developed by this laboratory for HILIC will continue to be based on such coatings.

ACKNOWLEDGEMENTS

I am indebted to W. V. Willis, M. C. Linder, D. R. McLachlan, C. A. Mizzen, M. Brasseur, J. F. Leykam, and H. E. Meyer for communicating results prior to publication, and to A. M. Fathy for donating the oligoglycosides. The advice and encouragement of Patricia Wirth-Nugent were crucial to the formulation of this study. Support came in part from NIH grant HL 36260 (NHLBI section).

REFERENCES

1 A. J. Alpert and P. C. Andrews, *J. Chromatogr.*, 443 (1988) 85.
2 D. L. Crimmins, J. Gorka, R. S. Thoma and B. D. Schwartz, *J. Chromatogr.*, 443 (1988) 63.
3 D. L. Crimmins, R. S. Thoma, D. W. McCourt and B. D. Schwartz, *Anal. Biochem.*, 176 (1989) 255.
4 D. H. W. Cheng, P.-J. Lee and K. W. K. Watt, presented at *7th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Washington, DC, Nov. 1987*, abstract 311.
5 P. C. Andrews, *Pept. Res.*, 1 (1988) 93.
6 D. L. Crimmins, D. W. McCourt and B. D. Schwartz, *Biochem. Biophys. Res. Commun.*, 156 (1988) 910.
7 T. D. Schlabach, J. C. Colburn, R. J. Mattaliano and S. Yuen, in T. E. Hugli (Editor), *Techniques in Protein Chemistry*, Vol. 1, Academic Press, New York, 1989, Ch. 48.
8 R. S. Hodges and C. T. Mant, University of Alberta, personal communication.
9 Y.-B. Yang and M. Verzele, *J. Chromatogr.*, 387 (1987) 197.
10 C. T. Mant, J. M. R. Parker and R. S. Hodges, *J. Chromatogr.*, 397 (1987) 99.

11 V. Carunchio, A. M. Girelli and A. Messina, *Chromatographia*, 23 (1987) 731.
12 J. C. Linden and C. L. Lawhead, *J. Chromatogr.*, 105 (1975) 125.
13 J. K. Palmer, *Anal. Lett.*, 8 (1975) 215.
14 F. M. Rabel, A. G. Caputo and E. T. Butts, *J. Chromatogr.*, 126 (1976) 731.
15 C. Brons and C. Olieman, *J. Chromatogr.*, 259 (1983) 79.
16 M. Verzele and F. Van Damme, *J. Chromatogr.*, 362 (1986) 23.
17 M. Verzele, G. Simoens and F. Van Damme, *Chromatographia*, 23 (1987) 292.
18 B. Bendiak, J. Orr, I. Brockhausen, G. Vella and C. Phoebe, *Anal. Biochem.*, 175 (1988) 96.
19 K. Koizumi, T. Utamura and Y. Okada, *J. Chromatogr.*, 321 (1985) 145.
20 A. J. Alpert, *J. Chromatogr.*, 266 (1983) 23.
21 A. J. Alpert, *J. Chromatogr.*, 359 (1986) 85.
22 D. Guo, C. T. Mant, A. K. Taneja, J. M. R. Parker and R. S. Hodges, *J. Chromatogr.*, 359 (1986) 499.
23 Z. Deyl, J. Hyanek and M. Horakova, *J. Chromatogr.*, 379 (1986) 177.
24 J. C. Robert, A. Soumarmon and M. J. M. Lewin, *J. Chromatogr.*, 338 (1985) 315.
25 M. C. McCroskey, J. R. Colca and J. D. Pearson, *J. Chromatogr.*, 442 (1988) 307.
26 H. E. Meyer, Ruhr-Universität Bochum, personal communication.
27 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
28 A. J. Alpert, *J. Chromatogr.*, 444 (1988) 269.
29 L. A. Th. Verhaar and B. F. M. Kuster, *J. Chromatogr.*, 234 (1982) 57.
30 P. Orth and H. Engelhardt, *Chromatographia*, 15 (1982) 91.
31 Z. L. Nikolov and P. J. Reilly, *J. Chromatogr.*, 325 (1985) 287.
32 P.-Å. Albertsson, *Partition of Cells and Macromolecules*, 3rd ed., Wiley, New York, 1986.
33 W. Müller and G. Kutemeier, *Eur. J. Biochem.*, 128 (1982) 231.
34 W. Müller, *Eur. J. Biochem.*, 155 (1986) 213.
35 W. Müller, *Eur. J. Biochem.*, 155 (1986) 203.
36 R. W. Schmid and Ch. Wolf, *Chromatographia*, 24 (1987) 713.
37 R. M. Smith and J. O. Rabuor, *J. Chromatogr.*, 464 (1989) 117.
38 H. Hosotbuso, S. Takahara and N. Taenaka, *J. Chromatogr.*, 432 (1988) 340.
39 H. Hosotbuso, *J. Chromatogr.*, 487 (1989) 421.
40 G. Johansson and M. Joelsson, *J. Chromatogr.*, 464 (1989) 49.
41 K. E. Bij, Cs. Horváth, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
42 B. Grego and M. T. W. Hearn, *Chromatographia*, 14 (1981) 589.
43 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 255 (1983) 125.
44 D. W. Armstrong and R. E. Boehm, *J. Chromatogr. Sci.*, 22 (1984) 378.
45 H. Engelhardt and H. Müller, *Chromatographia*, 19 (1984) 77.
46 R. S. Blanquet, K. H. Bui and D. W. Armstrong, *J. Liq. Chromatogr.*, 9 (1986) 1933.
47 R. J. Simpson, R. L. Moritz, E. E. Nice and B. Grego, *Eur. J. Biochem.*, 165 (1987) 21.
48 R. J. Simpson and R. L. Moritz, *J. Chromatogr.*, 474 (1989) 418.
49 R. J. Simpson, L. D. Ward, G. E. Reid, M. P. Batterham and R. L. Moritz, *J. Chromatogr.*, 476 (1989) 345.
50 D. W. Armstrong and W. Li, *Chromatography*, 2 (1987) 43.
51 S. M. Han and D. W. Armstrong, *J. Chromatogr.*, 389 (1987) 256.
52 H. L. Jin, A. M. Stalcup and D. W. Armstrong, *J. Liq. Chromatogr.*, 11 (1988) 3295.
53 D. W. Armstrong and H. L. Jin, *J. Chromatogr.*, 462 (1989) 219.
54 H. Engelhardt, H. Löw, W. Eberhardt and M. Mauss, *Chromatographia*, 27 (1989) 535.
55 J. Fausnaugh-Pollitt, G. Thevenon, L. Janis and F. E. Regnier, *J. Chromatogr.*, 443 (1988) 221.
56 M. Rubenstein, S. Rubenstein, P. C. Familletti, R. S. Miller, A. A. Waldman and S. Pestka, *Proc. Natl. Acad. Sci. USA*, 76 (1979) 640.
57 M. Rubenstein, *Anal. Biochem.*, 98 (1979) 1.
58 G. Helynck, B. Luu, J.-L. Nussbaum, D. Picken, G. Skalidis, E. Trifilieff, A. Van Dorsselaer, P. Seta, R. Sandeaux, C. Gavach, F. Heitz, D. Simon and G. Spach, *Eur. J. Biochem.*, 133 (1983) 689.
59 P. Boyot, E. Trifilieff, A. Van Dorsselaer and B. Luu, *Anal. Biochem.*, 173 (1988) 75.
60 P. Boyot, B. Luu, L. R. Jones and E. Trifilieff, *Arch. Biochem. Biophys.*, 269 (1989) 639.
61 M. H. Tadros, H. Zuber and G. Drews, *Eur. J. Biochem.*, 127 (1982) 315.
62 D. R. McLachlan and C. A. Mizzen, in preparation.
63 P. C. Andrews, in preparation.
64 J. F. Leykam, in preparation.

65 M. Brasseur, in preparation.
66 M. Colpan and D. Riesner, *J. Chromatogr.*, 296 (1984) 339.
67 R. R. Drager and F. E. Regnier, *Anal. Biochem.*, 145 (1985) 47.
68 L. W. McLaughlin and R. Bischoff, *J. Chromatogr.*, 418 (1987) 51.
69 R. Hecker and D. Riesner, *J. Chromatogr.*, 418 (1987) 97.
70 M. Sieber, in *Nucleosil 4000-7 PEI*, Macherey-Nagel, Düren, 1989, Fig. 6.