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High-Performance Liquid Chromatography of Biopolymers

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Summary. The ability to separate biological macromolecules with good resolution on liquid chromatographic columns has depended on the development of suitable packing materials. In size exclusion chromatography, molecules are separated by size on the basis of differential permeation of the packing. Ion exchange, hydrophobic interaction (or reversed-phase), and affinity chromatography are all surface-mediated separation methods, although they depend on different retention mechanisms. High-performance liquid chromatographic columns designed for biopolymers offer major advantages over conventional columns in both speed and resolving power. The exponential growth of literature on the high-performance separation of peptides and proteins in particular indicates that the technique will become the dominant form of column liquid chromatography.

Availability of pure substances for determining the structure and function of the enormous array of compounds encountered in biological systems has always been a problem in biochemistry. The fact that a cell may contain several thousand proteins, hundreds of RNA's, and multiple DNA and polysaccharide components makes the isolation of any single macromolecular species a challenging task. For this reason, the advancement of modern biochemistry and developments in macromolecular separations have been intimately linked. Contributions to our present understanding of biological systems derived from use of the analytical ultracentrifuge, electrophoresis, and column chromatography are examples of the impact of separation science on biochemistry. Unfortunately, many of the analytical techniques upon which life scientists have depended for the past two decades are labor-intensive, slow, and of low resolving power. In this article I will examine some of the recent attempts to circumvent these problems in column liquid chromatography of biological macromolecules.

Resolution of components in a liquid chromatographic system is dependent on their differential distribution between a solid stationary phase and a liquid mobile phase. Components of greater concentration in the mobile phase will elute from the column first, followed by those of lower concentration in the mobile phase. Differences in peak maxima between eluted components are a function of the relative difference in their distribution between the phases. Since biopolymers vary in size and shape, solubility, ionic characteristics, hydrophobicity, and affinity for other molecules, discrimination between any one or combination of these properties may serve as the basis for differential distribution within a separation system. Size exclusion, ion exchange, reversed-phase, hydrophobic interaction, and liquid affinity chromatography are column fractionation techniques designed to exploit one of these chemical or physical differences in biopolymers.

In addition to the relative difference in peak maxima, resolution is a function of peak width. Total resolution of two components will be easier if their peaks are very sharp because a smaller difference in peak maxima is required. It has been known for four decades (*1*) that reducing the particle size of chromatographic packing materials would result in the sharpening of eluted peaks. This is the result of two effects: shortening the distance through which a molecule must travel in its excursions into and out of support particles and limiting eddy diffusion in the particle bed (*2*). Implementation of this knowledge took so many years because (i) beds of very small particles require a high inlet pressure to achieve reasonable flow, (ii) precision flow high-pressure pumping systems were not available, (iii) the gel-type packing materials used in the separation of biopolymers could not tolerate high flow rates, and (iv) the technology for packing microparticulate beds had not been developed. By the late 1960's most of these problems had been solved for the separation of small molecules, and the name high-performance liquid chromatography (HPLC) was coined to describe rapid separations in microparticulate beds. Unfortunately, the high-performance packing materials of this era were not suitable for separation of biopolymers. Another 10 years elapsed before the emergence of commercial HPLC packings specifically designed for the separation of biopolymers.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) separates molecules by size based on differential permeation of the column packing. When molecules ranging in size from several hundred to several million daltons are swept through a bed of porous particles, their elution behavior suggests that there are two different volumes of liquid in the column; a volume

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(V_0) that is available to all molecules and a volume (V_i) that is differentially accessible based on solute size (*3*). These volumes correspond roughly to the interstitial and support pore volumes, respectively. Solute elution volume is described by the equation

$$V_e = V_0 + K_D V_i \quad (1)$$

where K_D is defined as the size exclusion distribution coefficient and corresponds to the fractional pore volume available to a molecule. Plots of the logarithm of solute molecular weight against K_D are roughly linear between K_D values of 0.15 and 0.80. The slope (m) of this calibration curve is related to the distribution of pore diameters in the column packing. As support pore distribution becomes more heterogeneous and m increases, the molecular weight range of the calibration curve is extended but the resolving power of the column decreases.

Resolving power of a size exclusion column is a function of four factors: (i) pore volume of the packing, (ii) packing density of the support, (iii) pore diameter distribution in the packing, and (iv) width of the chromatographic peaks (*3, 4*). The first three variables are related to inherent properties of the support. Peak width is a function of support particle size, how well the column is packed, mobile phase velocity, mobile phase viscosity, and solute diffusion coefficient.

The difference between peak maxima in SEC increases with pore volume. This is most easily illustrated with thyroglobulin and cytochrome c in Fig. 1. Thyroglobulin is sufficiently large that it elutes in the column void volume (*5*). Void volume of a column packed with spherical particles is approximately 37 percent of the total column volume, as opposed to 48 percent for a bed of irregularly shaped particles (*6*). The advantage of spherical particles is that less of the operating volume of the column is wasted on void volume. As pore volume of the column is increased, the cytochrome c elution volume will increase in direct proportion. Since these substances are the elution extremes—the first and last substances to emerge from an SEC column—an increase in the relative difference between their peak maxima leaves more room in the chromatogram for other components. Pore volume is a particularly important variable to monitor in high-performance size exclusion chromatography (HP-SEC) because it is often decreased in an effort to increase the mechanical strength of packings. Columns that are identical in all respects except pore volume can have greater than twofold differences in resolution.

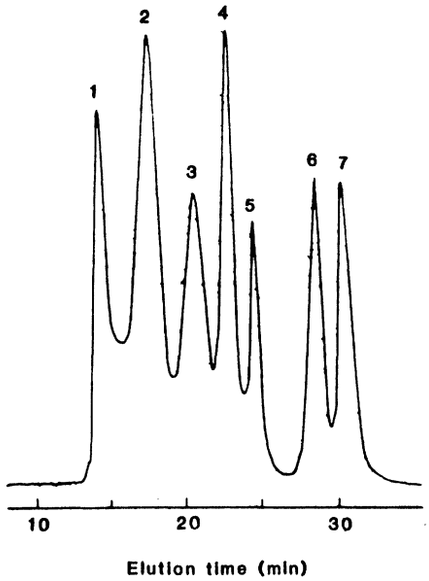

Fig. 1. Separation of a mixture of standard proteins: 1, thyroglobulin (porcine); 2, ferritin (horse spleen); 3, h-gamma globulin; 4, h-transferrin + h-albumin; 5, ovalbumin; 6, chymotrypsinogen; 7, cytochrome c. Column: G 3000 SW, 7.5 mm inner diameter, 30 cm + 50 cm with precolumn. Flow rate: 1 ml/min. Solvent: 1/15*M* potassium phosphate buffer, *p*H 6.8, containing 0.1*M* NaCl and 0.0006*M* sodium azide. Sample load: 20 to 80 μg; charge, 100 μl. Detector: ultraviolet, 280 nm, 0.01 absorbance unit full scale. [From (*5*)]

Mass transfer of solutes between the stationary and mobile phases has a strong influence on peak width in the chromatography of macromolecules. The contribution of particle size to mass transfer and eddy diffusion has already been noted. It is easily recognized that the diffusion of molecules between phases will require time. Although small particles decrease the time required for equilibration, increasing mobile phase velocity aggravates the problem. Rapid separations will be achieved at the expense of diminished mass transfer. Decreasing the rate of solute diffusion by increasing either mobile phase viscosity or solute molecular weight will also enhance the mass transfer problem.

It is most meaningful to estimate the resolving power of SEC columns by their ability to discriminate between molecules on the basis of size. This may be accomplished by using the molecular weight ratio (R_{MW}) of immediately adjacent peaks in an SEC chromatogram (*6*). For example, a column that is able to separate proteins of 100 and 50 kilodaltons (kD) to the extent that the peaks are resolved but just touching at their bases would have an R_{MW} of 2. Good HP-SEC columns produce an R_{MW} of approximately 2 with proteins of 30 to 80 kD in a 60-minute separation. Although the resolving power of conventional and HP-SEC columns is comparable, high-performance columns will accomplish a separation in much less time. Elution times of 10 minutes or less are common in HP-SEC. Since R_{MW} is proportional to mobile phase velocity, as shown in Fig. 2, very rapid separations suffer from loss of resolution. Figure 2 also reveals that R_{MW} is proportional to solute size. Resolution of high molecular weight species declines more rapidly at a high mobile phase velocity because of their lower rate of diffusion and mass transfer. Figure 2 implies that little is to be gained by using separation times of more than 2 to 4 hours with HP-SEC columns of 10-μm particle size.

The loading capacity of SEC columns is lower than in other chromatographic systems. For example, sample volume and mass loading capacities of a 7.8 by 300 mm SEC column would be approximately 100 μl and 1 mg, respectively (*8, 9*). In contrast, gradient-eluted ion exchange and reversed-phase columns of the same dimensions will load 10 to 15 times as much protein (*10, 11*).

Experience with conventional and high-performance columns suggests that the ideal packing material for HP-SEC should be (i) mechanically stable to a pressure of several hundred atmospheres, (ii) spherical, (iii) hydrophilic, (iv) non-ionic, (v) of high pore volume, (vi) available in particle sizes from 3 to 10 μm, (vii) available in pore diameters ranging from 50 to several thousand angstroms, (viii) of narrow pore distribution, (ix) chemically stable between *p*H 3 and 10, (x) nonbiodegradable, (xi) easy to pack, and (xii) inexpensive. Unfortunately, no support currently exists that has all of these properties.

Two types of support materials have been developed for HP-SEC of biopolymers; surface-modified silica matrices and totally organic gels. At present, the silica-based supports are distinctly superior. Since silanol groups at the surface of silica bear a weak negative charge, the surface must be modified before it is satisfactory for SEC. This is accomplished by derivatizing surface silanols with hydrophilic organosilanes such as glycerylpropyltrimethoxysilane (*12*) or *N*-acetylaminopropylsilane (*13*) to both neutralize the surface and make it hydrophilic. Although silylation greatly diminishes surface silanols on bonded phase silicas, steric factors make it impossible to totally derivatize all silanols (*14*). These residual silanols give all silica-

based SEC supports some anionic character (*6*). At low ionic strength, usually less than 0.1*M* salt, they will act as weak cation exchange materials.

The composition of most commercial SEC packings is proprietary. It is probable that the silica matrices used in commercial packings range from controlled porosity silica to aggregates of small surface-modified silica particles in organic polymers. The silica-based packings have excellent mechanical strength and may be used with all mobile phases that do not contain strong bases or oxidants. At *p*H values greater than 8, silica matrices begin to erode and column life is diminished (*4*, p. 52).

Several synthetic routes have been taken in the preparation of organic packings. One of the first approaches was to use ethylene glycol– or glycerol-derivatized methacrylates to prepare controlled porosity polymethacrylates (*15*). These materials have excellent mechanical strength but are sufficiently hydrophobic that they interact with many proteins. Such surface adsorption seriously diminishes the utility of this SEC packing. A second approach has been the preparation of agarose beads with 10 to 20 percent agarose (weight to volume) to increase mechanical strength (*16*). These packings appear to have excellent chromatographic properties and sufficient mechanical strength to withstand high mobile phase velocities. If they become commercially available, they could be strongly competitive with the current silica-based packings.

HP-SEC packing materials are sufficiently robust to tolerate a variety of mobile phases ranging from buffers to organic solvents. However, caution should be taken when changing mobile phases that salts are not precipitated in columns. Care must also be taken to wash columns sufficiently to remove the previous mobile phase. This may be very difficult in the case of detergents such as sodium dodecyl sulfate. The presence of particulate matter in mobile phases, in the form of dust or microorganisms, will plug column frits and the column itself. Pretreating solvents by the addition of 0.02 percent sodium azide and filtration circumvents these problems.

HP-SEC has been so widely applied in biochemistry that a meaningful discussion of its applications is not possible in this limited treatment. Instead, attention will be directed to situations where problems are anticipated. Pure SEC requires that there be no interaction between the column packing and the substances being separated. Hydrophobic and ionic

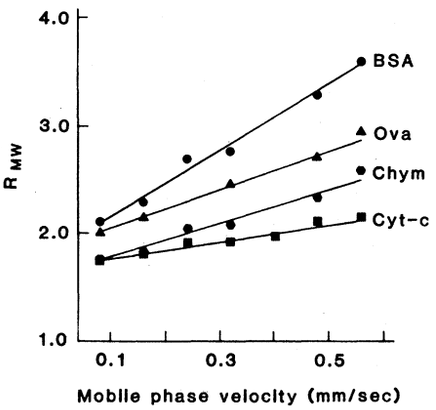

Fig. 2. Dependence of molecular weight resolution (R_{MW}) on mobile phase velocity. The column was a TSK SW 2000 operated with 0.1*M* phosphate buffer, *p*H 6.0. [From (*6*)]

interactions produce the major portion of all nonideal SEC behavior. By controlling the characteristics of the mobile phase, the experimenter seeks to prevent interactions between the column and solutes. For example, when both column and solutes have some hydrophobic character, the mobile phase must be of intermediate hydrophobic character to prevent their interaction (*16*). When the mobile phase is much more polar than both the column and the solutes, as in the case of mobile phases of high ionic strength, hydrophobic interactions between the column and solutes will be induced. In contrast, when the mobile phase is much less polar than the column and biopolymers, as in the case of propanol, acetonitrile, or tetrahydrofuran mobile phases, "normal phase" interactions occur between the column and solutes. Nonideal behavior of SEC columns is not always bad. The use of nonpolar solvents that enhance the interaction of polar proteins with SEC columns was effective in the purification of interferon (*17*).

Interactions between ionic column packings and ionic solutes are enhanced by mobile phases of low ionic strength and diminished by those of high ionic strength (*6*, *18*). As in the case of hydrophobic interactions, nonideal SEC behavior resulting from ionic interactions may be exploited in the resolution of biopolymers (*6*). Since all of the commercially available HP-SEC columns are anionic (*6*), some salt will be required in the mobile phase to prevent ionic interactions with the column. This is usually achieved with salt concentrations of 0.1 to 0.5*M*. The greatest problem arises when a column is both anionic and hydrophobic. Increasing the ionic strength to overcome ionic interactions can promote hydrophobic effects; decreasing it will have the reverse effect. Essentially, conditions may not be found where a column that is both ionic and hydrophobic will give perfect SEC separations.

HP-SEC has been carried out under both denaturing and nondenaturing conditions. Denaturing mobile phases may be used to eliminate shape differences between proteins, to disrupt associated proteins, and to solubilize polypeptides of limited water solubility. Sodium dodecyl sulfate and guanidine hydrochloride have been used successfully in HP-SEC to disrupt protein structure (*19*, *20*). Formic acid and triethylammonium phosphate in organic solvents have also been used to elute hydrophobic peptides from HP-SEC columns (*21*).

Nondenaturing eluants must be used when the objective is isolation of enzymes or other structurally labile species. This generally means elimination of organic solvents, detergents, and chaotropic agents from mobile phases. Fortunately, SEC columns will tolerate most common buffers, cofactors, sulfhydryl agents, and metal ions used in the purification of proteins, peptides, and polynucleotides.

Surface-Mediated Separation Modes

Ion exchange, hydrophobic interaction (also referred to as reversed-phase), and affinity chromatography are all considered to be surface-mediated separation modes even though the retention mechanisms are quite different. Some features of adsorption at surfaces are uniquely different for macromolecules and small molecules. These differences and their relation to macromolecular retention in chromatographic systems are examined below.

When one considers that biopolymers of even moderate size (50 to 100 kD) may have hundreds of residues capable of hydrophobic, ionic, or hydrogen-bonding interactions with surfaces, it is highly probable that they will be adsorbed at more than one site. Furthermore, such an adsorption process would be cooperative; that is, adsorption at one site would increase the probability of adsorption at other sites. Such multiple-site binding will require multiple molecules of displacing agents to cause macromolecular desorption (*22–24*). The equilibrium between a biopolymer, an adsorbing surface, and a displacing agent may be represented by

$$P_0 + zD_b \rightleftarrows P_b^n + zD_0$$

where P_0 is polymer in solution, D_0 is displacing agent in solution, D_b is displacing agent bound to the surface and P_b^n is protein bound to the support surface at n sites or residues. The z term is the number of molecules of displacing agent required to desorb a polymer from the surface; it may equal n, but in most cases will probably be larger. Expressing this process in terms of a formation constant (K_f) produces the expression

$$K_f = (P_b^n)(D_0)^z/(P_0)(D_b)^z \quad (2)$$

K_f should not be confused with the thermodynamic equilibrium constant in which activities of the species are used. Since the maximum loading capacity of a column in both the ion exchange and hydrophobic interaction modes is generally 1000 times greater than the average analytical load, D_0 is not significantly changed by an analytical sample. Furthermore, D_0 changes only a few percent in the elution of most biopolymers. These facts and other evidence (*25*) lead to the conclusion that $(D_b)^z$ is a constant. Since the distribution coefficient (K_i) in the ith mode is equal to the ratio P_b^n/P_0, Eq. 2 may be reduced to

$$K_i = K_{yi}/(D_0)^z \quad (3)$$

Combining this expression with that for the capacity factor (*26*), and setting the constants K_{yi} and ϕ (stationary- to mobile-phase volume ratio) equal to a new constant K_{zi}, produces an expression that relates chromatographic retention (k') to adsorption processes at surfaces:

$$k' = K_{zi}/(D_0)^z \quad (4)$$

From this expression it is seen that there is an exponential relation between chromatographic retention (k') and the number (z) of molecules of displacing agent required for desorption of a macromolecule. The facts that desorption curves for macromolecules become increasingly concave with increasing molecular weight and are different from those for small molecules support this description of surface-mediated separations.

It is surprising that in surface-mediated separations columns less than 5 cm long have more than 80 percent of the resolving power of 30-cm columns (*10, 27*). Apparently, multiple site interaction of biopolymers with surfaces makes their desorption curve so concave that columns separate more on the basis of a selective desorption process. Small analytical columns have the advantages that (i) solutes are diluted less upon elution and detection limits are lower, (ii) lower pressure is required for elution, (iii) they are easier to pack reproducibly, and (iv) they are less expensive.

Ion Exchange Chromatography

There can be little question that the new rigid high-performance ion exchange chromatography (HP-IEC) packings have introduced a new era in ion exchange chromatography of proteins. The resolution and speed of such high-performance columns is illustrated (Fig. 3) in the separation of lactate dehydrogenase isoenzymes on a silica-based weak anion exchange column (*28*). In addition to the high speed and resolution of these new materials, IEC generally permits increased resolution through differential manipulation of peak retention. To understand how this is possible, it is necessary to examine the retention process in IEC.

The degree of solute retention in IEC is governed largely by the strength of electrostatic interaction between solute and column. The force of attraction will depend on (i) the number of charges on the biopolymer that may interact simultaneously with the packing, (ii) the charge density on the packing, (iii) the ionic strength of the medium, and (iv) the type of ions in the medium. The chromatographer has three mobile phase variables with which to control retention and selectivity in IEC: (i) ionic strength, (ii) type of displacing ion or ions, and (iii) pH. The charge on both biopolymers and supports is often pH-dependent. The structural complexity of proteins makes their IEC retention the most complicated. Because of their amphoteric character, proteins have been chromatographed on both anion and cation exchange columns. In contrast, polynucleotides are generally separated on anion exchange columns because of the dominant influence of phosphodiester anions in the polymer backbone.

The most general technique for controlling retention in IEC is with the ionic strength of the displacing ions. The very narrow ionic strength range over which most proteins elute and the broad difference in their ionic characteristics make gradient elution the most useful technique for resolving protein mixtures (*29*). Columns are loaded at 0.01 to 0.02M salt and gradient-eluted up to 0.5 or 1M salt. The nature of displacing ions can also strongly influence IEC retention. The relative displacing power of various ions generally follows the Hoffmeister series (*29*), but there are numerous exceptions. At present, there is no predictable pattern to these exceptions.

An examination of the importance of pH in retention and resolution of proteins must be prefaced with a discussion of their charge characteristics. The amphoteric nature of polypeptides is revealed in their titration curve. Under acidic conditions, amino groups in polypeptides will be totally protonated while the ionization of carboxyl groups is repressed. As solution pH is increased, a point will be reached where the ratio of anionic to cationic groups is unity and the polypeptide is at its isoelectric point (pI). Further increases in solution pH will cause the polypeptide to acquire anionic character through ionization of carboxyls. Both the isoelectric point and the titration curve of a protein are unique.

It has generally been assumed that there is a strong correlation between the titration curve of a protein and IEC retention. Recent research has cast doubt on this assumption (*24*). Examination of IEC retention relative to net charge has failed to show complete correlation in more than 70 percent of the proteins tested. The principal deviation is that many proteins are retained on ion exchange columns at their pI, where they have no net charge. An additional deviation is that several pH units away from the pI some proteins show less IEC retention than would be expected from their net charge. Two factors have been identified that explain these results. First, charge asymmetry in polypeptides causes a heterogeneous distribution of charge at their surface. Even though the ratio of positive to negative charge is unity at the pI, a heterogeneous distribution of ionic groups within the polypeptide will cause differences in electrostatic potential at various points on the surface. This is important because the three-dimensional structure of a protein dictates the amount of its surface that may interact with the IEC matrix. Groups whose contact with the IEC surface is sterically limited will make little contribution to retention. For example, it was shown in the case of β-lactoglobulin that the number (z) of ionic groups interacting with the surface of an anion exchange packing may be much smaller than the net charge of the protein. This suggests that maximum resolution of components will be achieved at the pH where the relative difference in the number of ionic residues interacting with the surface is maximum. Unfortunately, there is no way to predict this pH a priori.

Four types of ionic stationary phases have been used in HP-IEC of biopolymers: (i) strong anion exchanging groups consisting of aliphatic quaternary amines, (ii) weak anion exchanging groups consisting of primary, secondary, or tertiary amines, (iii) a strong cation

exchanging sulfonic acid group, and (iv) a weak cation exchanging carboxyl group. The terms strong and weak are commonly used in reference to ion exchange materials but are misleading because they imply stronger and weaker retention. The term strong designates species that remain permanently ionized, while weak refers to weakly ionic groups. This means that surface charge density and therefore retention on weak ion exchange packings will vary considerably as mobile phase *p*H approaches the *pK* of the stationary phase. When the charge densities of both the column and the solute are *p*H-dependent, as in the case of proteins and weak ion exchange columns, retention is less predictable.

Both porous silica and organic resins have been used as supports for ion exchanging stationary phases. As in the case of SEC supports, surface silanols are sequestered during the application of organic coatings. Two procedures have been used to anchor organic coatings to the surface of silica: covalent bonding (*30–32*) and adsorption (*35*). In the first, the stationary phase is anchored either directly to the surface through a simple organosilane or to a polymer that is anchored to the surface through multiple organosilanes. Covalently bonded polymeric coatings have proved to be more stable because of multiple site attachment to the surface.

Adsorbed polymeric coatings are of equal stability and slightly easier to prepare. Adsorption of small ionic polymers of less than 1 kD followed by crosslinking produces a polymeric film that both sequesters silanols and provides an ion exchanging matrix (*33*). It has been demonstrated that ion exchange packings with a 300 Å pore diameter have the highest loading capacity and resolution for molecules in the range 30 to 100 kD (*34*). Larger molecules may require 500 or 1000 Å materials for optimum results. In the case of oligonucleotides of less than 50 bases, packings with a pore diameter of 100 Å give maximum resolution. The need for multiple pore sizes of IEC packings is apparent from these examples.

Organic ion exchange packings have also been used effectively in the separation of proteins. When the polymethacrylate matrices described above were derivatized with carboxymethyl and diethylaminoethyl groups, rapid separations of both proteins and nucleic acids were achieved with a resolution comparable or superior to that of their conventional gel-type counterparts (*35*). Carboxymethyl- and diethylaminoethyl-derivatized semirigid agarose gels have also been used to produce HP-IEC packings for proteins (*36*). The newest HP-IEC resins for proteins are the Monobead materials from Pharmacia Fine Chemicals AB. They are available in prepacked columns with a strong cation exchange group (Mono S), a strong anion exchange group (Mono Q), and weak anion exchanging groups (Mono P) for chromatofocusing. Pore diameter of the Monobead matrix is reported by the manufacturer to be 800 Å. These columns produce excellent separations of proteins in the *p*H range 3 to 11.

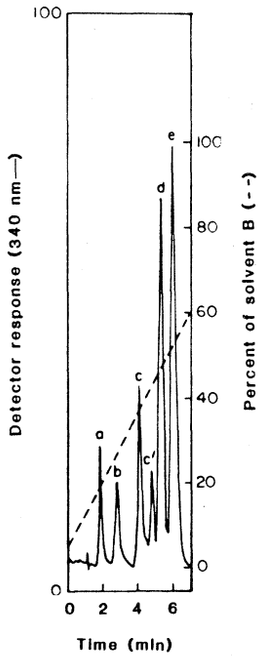

Fig. 3. Separation of LDH isoenzymes with a postcolumn enzyme detector. Column dimensions, 4 by 250 mm; packing, DEAE Glycophase/CPG (250-Å pore diameter, 5 to 10 μm particle size). Solvents: A, 0.025*M* tris *p*H 8.0; B, 0.025*M* tris, 0.2*M* sodium chloride, *p*H 8.0. Flow rate: 3 ml/min. Peak identification: a, LDH5; b, LDH4; c, LDH3; d, LDH2; e, LDH1.

HP-IEC packings currently used for polynucleotides are quite different from those described above for proteins. The polynucleotide resins are prepared by adsorbing trioctylmethylamine to the surface of polytrifluoroethylene particles (*37–39*). The hydrophobic nature of the support causes adhesion of the hydrophobic amine to its surface in buffers. Ionic strength gradient elution of these columns has been effective in the resolution of polynucleotides. The primary problems of these columns are leaching of the ion exchange coating and limited loading capacity.

Applications of HP-IEC are of two types: analytical and preparative. In many cases it is important to know the composition or purity of a polypeptide or polynucleotide sample. In the past, polyacrylamide gel electrophoresis (PAGE) has been a valuable tool for these analyses. It appears that HP-IEC may be equally suitable and much faster. In the case of water-soluble proteins, superior resolution of isoenzymes and variant hemoglobins has been observed with HP-IEC (*40–48*). Additional cases, where proteins that were considered pure after PAGE have been resolved into multiple components by HP-IEC, confirm that no single technique should be used as proof of purity and that HP-IEC is an equally valid technique for analyzing protein mixtures. Peptides may also be separated by HP-IEC, but separations are generally inferior to what can be achieved with HP-RPC.

HP-IEC is also of limited value as an analytical tool with polynucleotides. Although ion exchange separations of oligonucleotides with fewer than 30 bases may be achieved on the basis of chain length in 60 minutes (*49–51*), electrophoresis is superior with polynucleotides of 100 to several thousand bases (*52*). In addition, electrophoretic systems can analyze multiple samples simultaneously, and thus allow a greater number of analyses per day than HPLC.

The advantage of HP-IEC over electrophoresis in preparative separations is that it is inherently easier to scale up, the system may be used in repetitive separations, and sample recovery is simple. Preparative HP-IEC separations have been carried out on samples of less than 100 ng and more than 1 g. In the case of enzymes, recovery of enzyme activity generally equals or exceeds what has been achieved with conventional gel-type columns. Recoveries greater than 90 percent are common. Also, the elution protocol reported with a conventional gel-type column will usually work on the counterpart HP-IEC column, although differences in ligand density and chemical nature of the ion exchanging groups may cause proteins to elute at a different ionic strength. Most HP-IEC columns will tolerate sample loads of 2 mg per milliliter of column volume with no loss of resolution. In the relatively small number of cases where direct comparisons were made, HP-IEC columns gave much better purification than conventional columns (*32, 57*). Overloading columns with as much as 10 to 20 mg of protein per milliliter of column volume results in a loss of resolution, but components that are widely separated on analytical columns are still resolved.

The limited loading capacity and difficulty in carrying out preparative separa-

tions with electrophoretic systems prompted the development of chromatographic methods for the purification of polynucleotides. Although resolution of the RPC-5 ion exchange columns is inferior to that of electrophoretic systems, the ion exchange columns play an important role in the isolation of restriction enzyme digests of DNA (*39*).

Hydrophobic Interaction Chromatography

Separation of biopolymers by hydrophobic interactions between residues on the polymer and those on a column is practiced in several ways. When the support is porous silica derivatized with an alkyl silane at a ligand density of 2 to 4 μmole/m^2 and solutes are eluted with organic solvents, the technique is termed reversed-phase chromatography (RPC) (*58*). In contrast, separations achieved with descending salt gradients on alkyl-derivatized agarose have been termed hydrophobic interaction chromatography (HIC) (*59*). There can be little doubt that the retention mechanism is the same in both techniques. The principal difference, aside from the obvious one of the support matrix, is in the ligand density and therefore overall hydrophobic character of the chromatographic packing. In keeping with the concept that the name of a chromatographic process should in some way reflect what occurs in the column, hydrophobic interaction chromatography is clearly a better general name. Unfortunately, these techniques have been treated separately for so long that the trend cannot be reversed, and they will be discussed separately here.

The dominant factor controlling biopolymer retention by hydrophobic interaction is the hydrophobic contact area between solute and packing. Alkyl residues are only 7 Å apart on a well-coated RPC packing, which provides ample opportunity for multiple site adsorption of polymers (*60*). The distribution of hydrophobic residues in space and the number of residues that might interact with a surface are controlled by the primary, secondary, tertiary, and quaternary structure of the polymer. Considering that (i) hydrophobic interaction figures prominently in tertiary structure, (ii) biopolymers have a tendency to internalize hydrophobic residues within their three-dimensional structure, (iii) pairing agents are often required in the elution of biopolymers from RPC columns, (iv) mobile phases used to elute RPC columns at least partially disrupt the three-dimensional structure of biopolymers, and (v) there is not even general agreement on the retention mechanism for small molecules on RPC columns; an exact description of RPC of biopolymers is many years away.

Elution of proteins, peptides, and polynucleotides from RPC columns requires an organic solvent and often an acid. The organic solvent is needed to break down the intense hydrophobic interactions in the column and effect elution (*60*). In the case of bovine serum albumin, it has been shown that the number (z) of methanol molecules required for desorption is greater than 200 (*61*). This shows that biopolymers may be adsorbed to a surface at multiple sites and gives an idea of the contact surface area. Studies have indicated that each solute has a unique z value in a particular chromatographic system. Since acetonitrile and propanol are stronger displacing agents than methanol, they have smaller z values. Combinations of organic solvents have also been found to produce unique separations (*62*).

When the solute contains potentially ionizable species, the acid component of the mobile phase may contribute to retention by (i) influencing the ionic state of the solute, (ii) controlling ionization of surface silanols on the support, and (iii) forming ion pairs between cationic solutes and the acid. When a hydrophobic acid such as trifluoroacetic (*63, 64*) or heptafluorobutyric (*65*) acid is used, the ion-paired solute is more hydrophobic than the native solute and thus more strongly retained. With a very hydrophilic ion pair such as that formed between cations and phosphoric acid (*66*), solute retention will be less than that of the unpaired solute. Other ion-pairing agents such as alkyl sulfonic acids and alkyl amines have also been used to alter the selectivity of RPC columns and increase resolution (*60*). Amine pairing agents serve the dual role of ion-pairing with anions and negating the ionic effects of surface silanols. Through rechromatography on the same column and the use of multiple pairing agents, peptides have been purified from complex mixtures by RPC alone.

Use of acids in the mobile phase has been found to increase both recovery and resolution of many proteins. Trifluoroacetic acid (TFA) is the most popular one because it is an excellent solubilizing agent and allows detection of peptide bonds below 230 nm (*63, 64*). TFA is far less effective with membrane proteins. These very hydrophobic proteins require formic acid at a concentration of 5 to 50 percent (*68, 69*). Columns gradient-eluted from formic acid to formic acid in an organic solvent such as propanol or acetonitrile show substantially higher recovery with equivalent or superior resolution.

Organic solvents and other mobile phase modifiers also make secondary contributions to retention by altering three-dimensional structure. Disruption of hydrophobic and ionic interaction within polypeptides and polynucleotides will change their surface properties by altering tertiary and quaternary structure. The effect of three-dimensional structure on hydrophobic contact area has been noted above.

Retention of small peptides of less than 20 residues may be treated differently because they have little, if any, three-dimensional structure. The retention contributions of individual amino acids in small peptides can be quantitated and the values used to predict the retention of peptides of known structure (*67, 68*). As expected, lipophilic amino acids increase retention, cationic species decrease retention, and noncharged polar residues make little contribution to retention. A second approach to predicting retention is based on topological parameters and deals with end-group effects, positional isomers and analogs, and nearest-neighbor effects (*70, 71*).

The most suitable RPC columns to date have been organosilane-derivatized porous silica matrices. When an alkoxy- or chlorosilane containing an organic substituent is reacted with a silica surface, the organosilane is attached by siloxane bonding (*72*). This process causes the surface to acquire some of the properties of the organic moiety. Ethyl-, *n*-propyl-, *n*-butyl-, *n*-octyl-, *n*-octadecyl-, cyanopropyl-, alkylphenyl-, and diphenylsilanes applied in a monolayer or polymeric film have all been used successfully in the separation of biopolymers. In general, the shorter chain length monolayer coatings give the best performance in terms of resolution, loading capacity, and recovery when one must select a single column to chromatograph peptides, proteins, and polynucleotides (*73–75*).

Although a great deal of emphasis has been given to bonded phases, recent studies indicate that the support is more than a passive carrier of the bonded phase. Both pore diameter and nature of the silica surface play a role in recovery and resolution of very hydrophobic polypeptides. Macroporous (> 300 Å) supports have yielded superior resolution and recovery of polypeptides and proteins (*75–77*). However, the nature of the support material itself seems to be even more important. Of a series of five 300-Å

supports from different manufacturers, only two were of value in RPC of biopolymers (*78*). It has been suggested that some of the macroporous supports have a slightly more hydrophilic character than others (*79*).

Polystyrene-divinylbenzene resins have also been reported to be useful for RPC of peptides (*80*). The principal advantage of these supports is that they may be used in the *p*H range 2 to 12. Selectivity of the matrix has been reported to be similar to the alkyl silane bonded phase supports. The 80-Å pore diameter of this material may be a limitation in the separation of higher molecular weight species.

Weakly hydrophobic packings for high-performance hydrophobic interaction chromatography have also been reported (*81–83*). Alkyl chains in the C_2 to C_5 range appear to be of the greatest utility when applied to supports at low ligand density. Retention and elution are achieved by introducing the biopolymers at a high salt concentration and running a descending gradient to low ionic strength. The advantage of HP-HIC is that recovery of biological activity is higher than with RPC. It is also interesting that elution order from the two types of columns is different. For example, lysozyme is strongly retained relative to ovalbumin on HP-HIC columns, whereas on RPC columns the elution order is reversed (*83*). Although lysozyme does not have a particularly high concentration of hydrophobic amino acids, their concentration at the surface is high. In the HP-HIC system, the three-dimensional structure remains intact during separation. Presumably, differences in the number of hydrophobic residues at the surface of biopolymers are responsible for retention.

Application of RPC to peptide separations has revolutionized peptide chemistry. There was no comparable conventional counterpart to RPC. Single amino acid substitution is usually detectable in peptides of less than 20 amino acids. However, it is worth noting again that there may be broad differences in the way various RPC packings separate polypeptides (*11, 77*). These differences become particularly prominent with higher molecular weight hydrophobic peptides. Recovery from RPC columns is generally very high, but cases where problems occur should be noted. Both very hydrophobic and very basic peptides may be difficult to recover. Recovery of peptides containing over 80 percent hydrophobic residues was substantially enhanced by using 5 percent formic acid in the mobile phase (*65*). Macroporous columns have also been useful with hydrophobic CNBr fragments (*75, 76*). Triethylamine phosphate was reported to enhance the recovery of basic peptides (*84*). All recovery problems become more acute at very small sample loads. Since column length and volume make little contribution to resolution, very small columns will increase sample recovery by minimizing the amount of column packing encountered by a solute.

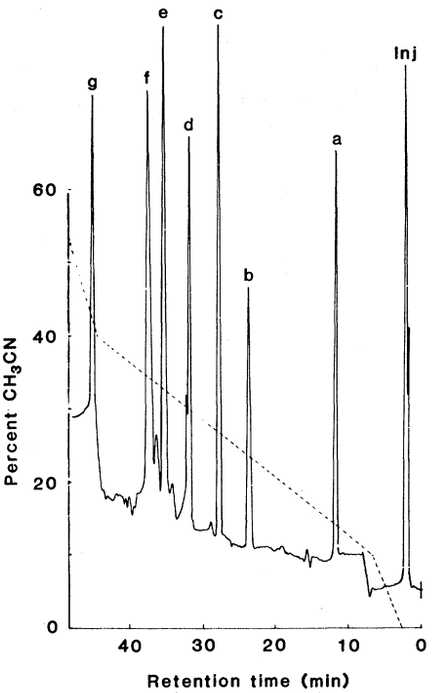

Fig. 4. Chromatogram of protein standards separated at ambient temperature on Hypersil ODS. Primary solvent: 0.1*M* phosphate buffer, *p*H 2.1; secondary solvent, acetonitrile. Peaks: a, tryptophan; b, cobra neurotoxin 3; c, ribonuclease A; d, insulin (complete molecule); e, cytochrome c; f, lysozyme; g, myoglobin.

Although protein fractionation by RPC has seen more limited application, it is still a very high speed, high-resolution technique, as shown in Fig. 4 (*85*). Initial recovery problems have been overcome by using the techniques outlined above for recovery of hydrophobic peptides. Macroporosity and source of the silica are even more important with proteins than with peptides (*75, 78*). With the proper column, proteins of greater than 100 kD are now routinely chromatographed on RPC columns by gradient elution. The most popular mobile phase for proteins is a gradient ranging from 0.1 percent TFA to 0.1 percent TFA in propanol or acetonitrile. Use of such harsh mobile phases will disrupt the three-dimensional structure of most biopolymers and poses the risks of (i) loss of enzyme activity, (ii) loss of weakly associated cofactors, (iii) fractionation of multiple subunit proteins into their component parts, and (iv) cleavage of covalent bonds in some labile species. Obviously, RPC will be of limited utility in enzyme purification.

Oligonucleotides of up to 11 bases have also been fractionated by RPC (*86, 87*) where retention is influenced by (i) oligomer chain length, (ii) hydrophobicity of derivatizing groups, (iii) chemical nature of the bases and their relative ratio, and (iv) the number of phosphate groups in the oligomer. Paired-ion separations with alkylammonium additives have also been used effectively with oligonucleotides of up to 16 bases (*88*).

Liquid Affinity Chromatography

The term liquid affinity chromatography (LAC) has been used to describe separations that are based on the bioaffinity of a protein for an immobilized ligand similar in structure to the natural ligand. Through the use of high-speed pumping systems and porous silica supports with covalently bonded ligands, LAC separations have been achieved in a few minutes. Very rapid analyses are aided by packings with small particle sizes and supports of either small or very large pore diameter, both of which maximize mass transfer and minimize band spreading. Loading capacity is also influenced strongly by pore diameter because the ratio of pore size to protein size determines the support surface area available to a protein. Basically two types of bioaffinity have been exploited in high-performance LAC; antigen-antibody binding and the adenine-triazine dye affinity for the so-called adenine binding cleft of oxidoreductase and kinase enzymes. Although the kinetics of the equilibration process in LAC is slow and plate height is low in many cases, manipulation of the mobile phase with step gradients allows separations in a few minutes. This is possible because of the enormous selectivity of the LAC system. In terms of applications, there is no reason to believe that HP-LAC systems will show different behavior than their conventional counterparts. The strategy for preparing LAC columns and eluting them should be similar to that used on conventional gel-type columns.

The Future

High-performance liquid chromatography of biopolymers is a powerful new technique that will change the way life scientists conceive and solve research

problems. The current generation of HPLC columns for biopolymers offers major advantages over conventional columns in both speed and resolving power. The fact that all of the high-performance columns, except those used in RPC, retain macromolecules by the same mechanism as their conventional counterparts is an added advantage. This will make it possible to simply substitute high-performance columns in established purification schemes with little change in protocol. The exponential growth of literature related to the high-performance separation of peptides and proteins in particular makes it easy to predict that use of the technique will continue to grow until it is the dominant form of column liquid chromatography.

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