*Journal of Chromatography*, 218 (1981) 535–546
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,154

# ASSESSMENT AND OPTIMIZATION OF SYSTEM PARAMETERS IN SIZE EXCLUSION SEPARATION OF PROTEINS ON DIOL-MODIFIED SILICA COLUMNS

P. ROUMELIOTIS and K. K. UNGER*
*Institut für Anorganische Chemie und Analytische Chemie, Johannes-Gutenberg-Universität, 6500 Mainz (G.F.R.)*

## SUMMARY

On diol-modified silica columns the retention of proteins is governed by a size exclusion effect, but superimposed on this are some secondary effects, *i.e.*, ionic and diol-ligand interactions which can be controlled and adjusted reproducibly by varying the eluent composition. The eluent composition also affects the column efficiency and peak shape. Both dependences can be employed to obtain a better resolution of proteins than can be expected from size exclusion alone.

## INTRODUCTION

The rapid separation of native biopolymers by high-performance liquid chromatography (HPLC) has attracted great interest as a means for both identification and isolation. Depending on the nature of the biopolymer and the purpose of the separation, three kinds of HPLC phase systems are commonly employed: reverse phase packings with ternary solvent mixtures, ion exchangers with buffers as eluents and size exclusion packings based on polar modified silicas with buffers[1]. Of these size exclusion chromatography (SEC) is most frequently carried out prior to the other separation modes, *e.g.*, to isolate fractions of a certain molecular weight. For SEC of biopolymers a number of bulk materials and also prepacked columns were developed. These have recently been examined and compared for column selectivity and performance[2].

The molecular weight (MW) selectivity of these columns is expressed in terms of a calibration plot of log MW *vs.* elution volume, $V_e$, of solute, or by the distribution coefficient, $K_D$, of solute, taking values between 0 and 1 (ref. 3). Although the calibration curve is fairly linear for most columns investigated at a specific eluent composition, deviations are observed in some MW ranges when plotting all data in the log MW *vs.* $V_e$ curve. This indicates that, in addition to size exclusion, other mechanisms are operating in the elution of biopolymers, and hence predictions of MW of unknown solutes based on $V_e$ may be highly erroneous. This particular behaviour of biopolymeric solutes arises from the fact that variation of eluent composition has a significant influence on the charge and structure of solutes, as well as on the surface of the packing[4].

0021-9673/81/0000–0000/$02.50 

The objective of this study was to elucidate the various contributions from non-size-exclusion effects to the elution volume of proteins on diol-modified silica columns. This enables an assessment of the most dominant mobile phase parameter controlling resolution and optimization of a separation. It will be demonstrated that the operation of several retention mechanisms in SEC of biopolymers may result in a resolution superior to that of pure SEC.

## EXPERIMENTAL

Two packings were employed in this study: LiChrosorb® Diol, particle diameter, $d_p$, 5 $\mu$m (E. Merck, Darmstadt, G.F.R.), and a diol-silica prepared by ourselves. The latter was obtained by modifying a 5-$\mu$m angular silica (mean pore diameter 30 nm) with 1,2-epoxy-3-propoxypropyltrimethoxysilane (Dynamit Nobel, Köln, G.F.R.), followed by treatment in 0.1 *N* sulphuric acid at 80°C to convert the epoxy into diol groups. The surface concentration of bonded groups was 4.8 $\pm$ 0.3 $\mu$mol/m$^2$ for both products, assuming a bifunctional reaction. The monolayer capacity was calculated at 2.65 $\mu$mol/m$^2$, and thus the values indicate some degree of polymerization of the bonded layer[5].

Columns (250 × 6 mm) were packed according to the high viscosity method using a 10% (w/w) slurry of paraffin–tetrachloromethane (50:50, v/v). The eluents were electrolytes and buffers, all of reagent grade (E. Merck). Standard proteins and dextrans were purchased from Boehringer (Mannheim, G.F.R.) and Serva (Heidelberg, G.F.R.). The chromatograph, a Hewlett-Packard Model 1084 A, was fitted with a UV photometer operated at a wavelength of 254 nm.

The electrophoretic velocities, $v$, of suspensions of the packing in various eluents were determined in a quartz cell (100 × 470 × 0.5 mm) using an Elphor VaP 11 (Bender & Hobein, München, G.F.R.). The zeta potential was calculated by the Helmholtz–Smoluchowski equation

$$\zeta = \eta v/\varepsilon_e \varepsilon_0 E$$

where $\eta$ = viscosity, $E$ = field strength, $\varepsilon_e$ = dimensionless dielectric constant and $\varepsilon_0 = 8.85 \cdot 10^{-12}$ cm$^{-1}$.

## RESULTS AND DISCUSSION

### *Elution of proteins on diol-modified silica columns*

Diol-modified silicas were chosen to examine the recovery, separation and isolation of proteins[6–8] on account of their favourable surface properties. Most of the studies were carried out in eluents of around pH 7.0 and appropriate ionic strength. An optimum linear fit of the calibration curve could be achieved for particular eluent compositions. However, when plotting all the $V_e$ values obtained at various eluent compositions on the log MW *vs.* $V_e$ diagram a large scattering was observed. Fig. 1 exemplifies the situation on a LiChrosorb Diol column (250 × 6 mm) with eluents of various pH ranging from 5.4 to 9.5 and almost constant ionic strength. Similar scattering occurs for the $V_e$ values of proteins at different buffer and electrolyte concentrations and constant pH. Thus, the data demonstrate that the mechanism in this

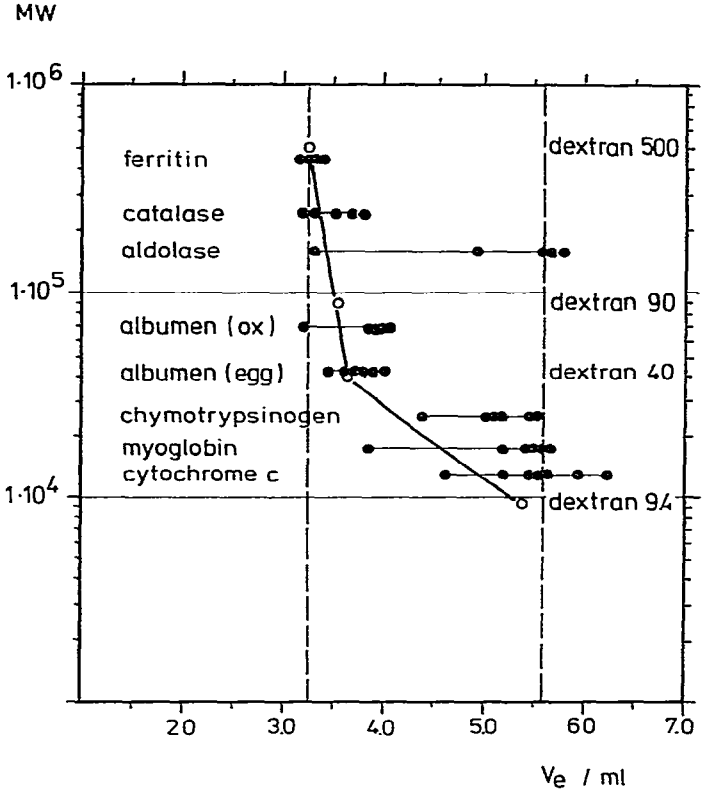


Fig. 1. Calibration curve for proteins and dextrans on a LiChrosorb Diol column (250 × 6 mm) with eluents of various pH (data taken from Table II).

particular phase system is not just pure size exclusion, additional effects being involved. Further, it is clear that all proteins are generally eluted between the interstitial volume, $V_0$, of the column (= 3.2 ml) and $V_0 + V_i$ (= 5.7 ml), $V_i$ being the internal column volume. This indicates that size exclusion is the controlling effect, while the other possible mechanisms contributing to $V_e$ are second-order effects.

Pure size exclusion is basically an entropy controlled process[3], giving a distribution coefficient $K_D \approx \exp(-\Delta S_0/R)$. Thus, peak retention may be expected to be temperature-independent. On the other hand, mechanisms based on solute–stationary phase interaction in liquid–solid chromatography (LSC) are enthalpy controlled processes. The distribution coefficient $K_{LSC}$ is proportional to $\exp(-\Delta H/RT)$. $\Delta H$ is usually negative and $K_{LSC}$ is dependent on temperature.

It is conceivable that solute–stationary phase interactions of biopolymers may take place when the solutes permeate the pore structure of the packing and hence gain access to the internal surface area. When the diol-modified silica surface is exposed to a buffered eluent two surface sites can be distinguished: ionic $SiO^-$ groups and polar $-(CH_2)_3OCH_2\underset{\displaystyle OH}{\underset{|}{C}}H\underset{\displaystyle OH}{\underset{|}{C}}H_2$ groups which are undissociated. Deprotonation of silanol groups, with $pK_a$ 6.8 (ref. 9), depends on the pH. The $SiO^-$ groups thus formed are

simultaneously involved in ion-exchange reactions with cations of the electrolyte. Positively charged basic proteins are attracted to negative surfaces while negatively charged acidic proteins are repelled. As a first approximation, solute–surface interactions can be divided into two types: $SiO^-$–solute, *i.e.*, ionic, and diol-ligand–solute, which may be of a hydrophobic nature. Supposing that both interactions contribute independently to retention, the following assumption can be made:

$$V_e \text{ (observed)} = V_{e(SE)} + V_{e(II)} + V_{e(DL)}$$

Here $V_e$ represents pure size exclusion, $V_{e(II)}$ the ionic and $V_{e(DL)}$ the diol-ligand interactions for a given solute.

The next problem to be solved is to find approaches which permit an estimation of the various contributions to $V_e$ (observed). This can be done in the following ways:

(i) by studying the elution behaviour of those molecules which appear to be controlled mainly by one of the three mechanisms, *e.g.*, uncharged dextrans as reference substances for pure size exclusion effects, small monomeric ionic solutes for ionic interactions;

(ii) by systematically varying those parameters of the phase system which are assumed to affect one specific type of interaction, *i.e.*, column temperature, pH, type, valence and concentration of the eluent, etc.

Furthermore, changes in the surface charge of diol-modified silica particles suspended in the various eluents might be monitored by measuring the electrophoretic mobility, $v$, and the zeta potential, $\zeta$.

*Estimation of size exclusion effects in the elution of proteins on diol-modified silicas*

Standard dextrans were applied in order to assess the extent of pure size exclusion relative to the other effects contributing to $V_e$ (observed) of proteins, and the temperature dependence of $V_e$ was examined both for proteins and dextrans. Three ionic test solutes, sulphanilic acid (p$K$ 1, 3.25), phenylalanine (p$K$ 2.58, 9.24) and benzoic acid (p$K$ 4.20), were included in the study. The results for two diol packings and different eluent compositions are listed in Table I. $V_e$ of proteins decreases slightly when the column temperature is raised from 25°C to 50°C, with the exception of aldolase and chymotrypsinogen, where the opposite takes place. This decrease may be understood in terms of weaker adsorption interactions between the solutes and the surface. The behaviour of aldolase and chymotrypsinogen is more difficult to interpret. These compounds either undergo conformational changes with temperature, or the adsorption interactions are associated with a positive enthalpy. The ionic solutes are eluted close to $V_i + V_0$ and hence may be considered as totally permeating solutes. The change in $V_e$ with column temperature indicates that adsorption interactions are involved in the retention. It is of interest in this context that a so-called enthalpic exclusion of these solutes, as observed on reversed-phase columns[10], does not appear to take place in this phase system.

Table I also shows that (i) the elution volumes of dextrans remains unaffected by variation in the column temperature, and (ii) the elution volumes of proteins of comparable molecular weight to dextrans, *e.g.*, albumen (egg) and dextran 40, are slightly higher. This demonstrates clearly that dextrans are eluted by a pure size

TABLE I

EFFECT OF COLUMN TEMPERATURE ON THE ELUTION VOLUMES OF PROTEINS, DEXTRANS AND IONIC SOLUTES

| *Solute* | *MW* | *LiChrosorb Diol, $d_p$ = 5 μm* | | | | *Diol-modified silica (30 nm), $d_p$ = 5 μm.* | |
|---|---|---|---|---|---|---|---|
| | | *Sörensen phosphate buffer, pH 7.4, + 0.02 m NaCl* | | *Sörensen phosphate buffer, pH 7.4* | | *Sörensen phosphate buffer, pH 7.4, + 0.02 m $NaNO_3$* | |
| | | *25°C* | *50°C* | *25°C* | *50°C* | *25°C* | *50°C* |
| Ferritin | 440,000 | 3.33 | 3.19 | 3.26 | 3.22 | 3.20 | 3.10 |
| Catalase | 240,000 | 3.40 | 3.30 | 3.38 | 3.32 | 3.60 | 3.54 |
| Aldolase | 158,000 | 3.44 | 4.00 | 3.46 | 5.42 | 3.78 | 5.32 |
| Albumen (bovine) | 68,000 | 3.56 | 3.40 | 3.52 | 3.49 | – | – |
| Albumen (egg) | 45,000 | 3.70 | 3.58 | 3.62 | 3.58 | 3.60 | 3.54 |
| Chymotrypsinogen | 25,000 | 5.30 | 5.70 | 5.74 | 6.14 | – | – |
| Myoglobin | 17,000 | 5.12 | 4.94 | 5.14 | 5.04 | – | – |
| Sulphanilic acid | 174 | 5.60 | 5.38 | 5.54 | 5.14 | 5.48 | 5.28 |
| Phenylalanine | 165 | 6.34 | 6.18 | 6.42 | 6.14 | 6.08 | 5.60 |
| Benzoic acid | 122 | 5.98 | 5.64 | 5.92 | 5.50 | 5.78 | 5.54 |
| Dextran 500 | 500,000 | 3.23 | 3.20 | 3.25 | 3.20 | 2.84 | 2.80 |
| Dextran 90 | 90,000 | 3.28 | 3.26 | 3.28 | 3.26 | 3.12 | 3.12 |
| Dextran 40 | 40,000 | 3.50 | 3.45 | 3.46 | 3.50 | 3.32 | 3.28 |
| Dextran 9.4 | 9400 | 5.40 | 5.30 | 5.40 | 5.30 | 5.28 | 5.28 |

exclusion mechanism while proteins undergo some additional interactions. The extent of the contribution of size exclusion to $V_e$ of proteins may be quantitated by subtracting the $V_e$ of dextrans with the same molecular weight, or more correctly the same hydrodynamic volume.

*Ionic effects on the elution of proteins*

It may be assumed that the small-size ionic substances used in this study are retarded only by ionic and possibly by additional ligand–solute interactions on diol-modified silicas. Furthermore, the strongest influence on solute retention due to ionic interactions in this phase system will arise from changes in the charge and charge distribution, respectively, of interacting entities, *i.e.*, through variation in pH and/or electrolyte concentration of the eluent. Table II lists the elution volumes of proteins and ionic reference substances measured over a wide range of eluent pH at almost constant ionic strength. For a meaningful discussion, Fig. 2 also needs to be considered, which, at the same eluent compositions as in Table II, presents the dependence of the zeta potential of suspensions of the LiChrosorb Diol packing on the pH of the eluent. It is seen that the negative charge on diol-silica particles increases with pH. The values are comparable to those obtained by Knox *et al.*[10] on ODS-Hypersil, but are slightly lower than those measured by Kerner and Leiner[11] on Aerosil and phenylated Aerosil. It is somewhat surprising that, even when subjected to modification, the electrophoretic mobility of silica particles can still be monitored. Pre-

TABLE II

EFFECT OF ELUENT pH ON THE ELUTION ($V_e$ IN ml) OF PROTEINS AND IONIC SOLUTES

The pH was adjusted by adding hydrochloric acid or ammonia to a 0.1 *m* solution of sodium acetate. Column: LiChrosorb Diol, $d_p$ = 5 μm, 250 × 6 mm.

| *Protein* | *pH of the eluent* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *9.49* | *7.56* | *6.48* | *6.08* | *5.89* | *5.67* | *5.42* |
| Ferritin | 3.16 | 3.24 | 3.26 | 3.34 | 3.34 | 3.35 | 3.38 |
| Catalase | 3.20 | 3.31 | 3.50 | 3.68 | – | 3.65 | 3.76 |
| Aldolase | 3.29 | 4.92 | 5.60 | 5.68 | – | 5.70 | 5.80 |
| Albumen (bovine) | 3.22 | 3.54 | 3.86 | 3.86 | 3.88 | 3.90 | 4.06 |
| Albumen (egg) | 3.42 | 3.62 | 3.78 | 3.84 | 3.86 | 3.90 | 4.00 |
| Chymotrypsinogen | 4.40 | 5.20 | 5.60 | 5.50 | 5.54 | 5.14 | 5.10 |
| Myoglobin | 3.86 | 4.94 | 5.58 | 5.54 | 5.62 | 5.46 | 5.50 |
| Cytochrome *c* | 4.74 | 5.20 | 5.48 | 5.94 | 6.24 | 5.64 | 5.54 |
| Sulphanilic acid | 5.00 | 5.32 | 5.46 | 5.64 | 5.64 | 5.68 | 5.70 |
| Benzyltrimethyl-ammonium chloride | large | 17.90 | 10.58 | 9.84 | 9.56 | 9.40 | 9.00 |
| Phenylalanine | 5.28 | 6.18 | 6.06 | 6.12 | 6.12 | 6.14 | 6.10 |
| Benzoic acid | 4.84 | 5.62 | 6.12 | 6.28 | 6.34 | 6.54 | 6.84 |

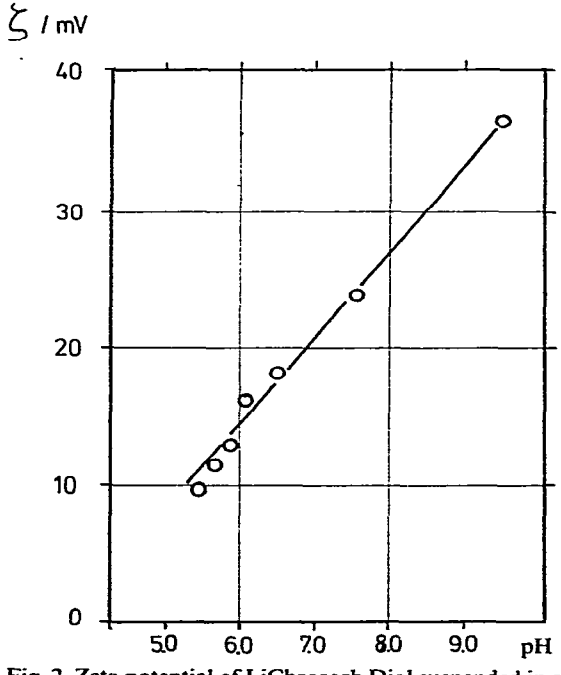


Fig. 2. Zeta potential of LiChrosorb Diol suspended in eluents of various pH (see Table II) as a function of the pH of the suspension.

sumably this should be attributed to the fact that a high population of silanols remains unaffected by surface reaction.

The data in Table II indicate that $V_e$ of most proteins decreases steadily with increasing pH from 5.4 to 9.5. The last three basic proteins, chymotrypsinogen, myoglobin and cytochrome C, show a slight maximum in the $V_e$ *vs.* pH dependence, but then $V_e$ values in alkaline media are always lower than in weak acidic media. Depending on the isoelectric pH of the protein, the relative ratio of negative to positive charges will increase with eluent pH. The negative surface charges will also increase, so that ionic repulsion becomes dominant and $V_e$ decreases. Ionic repulsion forces, however, are not strong enough to exclude all proteins totally from the internal pores. For all protein solutes, $V_e$ is observed to vary within $V_0$ and $V_0 + V_i$, indicating that ionic interactions are of secondary importance. This does not hold for small-size ionic solutes, as the whole internal surface is accessible for interactions. For phenylalanine, sulphanilic acid and benzoic acid, $V_e$ falls with increasing pH, the effect being most pronounced for benzoic acid. According to Knox *et al.*[10] the retention of benzoic acid above $V_i + V_0$ ($k' > 0$) can be explained by considering the retentivities of both forms of the acid, HA and $A^-$. Of all the solutes, retardation is highest for benzyltrimethylammonium cation. A change from pH 5.4 to 7.6 leads to a doubling of $V_e$, mainly as a result of the increasing number of negative surface charges on the diol-modified silica (see Fig. 2).

Ionic interactions between charged species are known to be controlled by the type, valence and concentration of the electrolyte. By changing these parameters the properties of the silica surface and the solutes are affected in various ways: the configuration of proteins is sensitive to electrolyte concentration; metal ions may form complexes with proteins; adsorbed anions influence the degree of hydration of proteins according to the chaotropic series; depending on the electrolyte concentration, a double layer around the protein solute is built up, changing the charge and charge distribution. In the same way the properties of the silica surfaces varied according to the electrolyte, via deprotonation, ion exchange, adsorption, etc.

Schmidt *et al.*[6] studied the effect of eluent ionic strength on the elution volume of proteins and found typical dependences for basic, acidic and neutral solutes. We believe that ionic strength is too simple a parameter to permit specification and elimination of single effects caused by the type of cation and anion, by the valence, etc. Assuming ionic strength, $\mu$, to be the determining parameter, all $V_e$ values of a given protein at the same level of $\mu$ should be equal. This, however, is not the case. Another feature is that at low ionic strength, $\mu < 0.1$, $\mu$ is adjusted by dilution of the buffer, while at $\mu > 0.1$ discrete $\mu$ values are obtained by adding a certain amount of electrolyte to the buffer. Table III lists the elution volumes of proteins at a wide range of electrolyte compositions. The eluent compositions are arranged in the order of decreasing zeta potential, which was measured in a suspension of the eluent with diol-modified silica. It is apparent that no correlation can be established between the zeta potential of silica particles and the retention of proteins at a constant pH of 7.4, leading to the interpretation that the observed deviations cannot be related to purely ionic interactions but also to other, *e.g.*, hydrophobic, interactions.

The data call for discussion of the relationship between $V_e$ and the following quantities: type of cation ($Li^+$, $Na^+$, $K^+$, $Cs^+$) at constant anion and concentration; type of anion ($NO_3^-$, $ClO_4^-$, $Cl^-$) at constant cation and concentration; the valence of

TABLE III

EFFECT OF IONIC TYPE, VALENCE, CONCENTRATION, IONIC STRENGTH AND ZETA POTENTIAL ON THE ELUTION VOLUME OF PROTEINS AND IONIC SOLUTES

Column: 250 × 6 mm, packed with LiChrosorb® Diol.

| | *$V_e$ (ml) in Sörensen phosphate buffer, pH 7.4* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *+0.02 m LiCl* | *+0.02 m $NaNO_3$* | *+0.08 m $NaClO_4$* | *+0.02 m CsCl* | *+0.02 m KCl* | *+0.0067 m $MgCl_2$* | *+0.02 m NaCl* | *+0.0067 m $Na_2SO_4$* | *+0.02 m $NaClO_4$* | *+0.0267 m $Na_2SO_4$* | *+0.08 m NaCl* | *+0.02 m $MgCl_2$* |
| *$\mu$* | *0.1008* | *0.1008* | *0.1618* | *0.1008* | *0.1008* | *0.1008* | *0.1008* | *0.1008* | *0.1008* | *0.1608* | *0.1608* | *0.1408* |
| *$\xi$ (mV)* | *−35.53* | *−31.87* | *−30.17* | *−28.48* | *−28.41* | *−27.85* | *−27.85* | *−27.85* | *−26.44* | *−25.38* | *−22.07* | *−19.88* |
| *Protein* | | | | | | | | | | | | |
| Ferritin | 3.30 | 3.32 | 3.32 | 3.30 | 3.31 | 3.28 | 3.33 | 3.34 | 3.34 | 3.30 | 3.32 | 3.34 |
| Catalase | 3.40 | 3.46 | 3.42 | 3.48 | 3.43 | 3.40 | 3.40 | 3.42 | 3.42 | 3.42 | 3.43 | 3.46 |
| Aldolase | 3.58 | 3.60 | – | 3.58 | 3.59 | 3.58 | 3.44 | 3.52 | 3.54 | – | – | 3.60 |
| Albumen (bovine) | 3.68 | 3.68 | 3.66 | 3.70 | 3.76 | 3.66 | 3.56 | 3.64 | 3.64 | 3.80 | 3.82 | 3.88 |
| Albumen (egg) | 3.70 | 3.75 | 3.76 | 3.76 | 3.77 | 3.74 | 3.70 | 3.72 | 3.72 | 3.80 | 3.78 | 3.80 |
| Chymotrypsinogen | 4.86 | 4.82 | 4.60 | 4.80 | 4.82 | 4.90 | 5.30 | 5.04 | 4.88 | 4.54 | 4.68 | 4.88 |
| Myoglobin | 5.80 | 4.80 | 4.60 | 6.06 | 6.14 | 5.92 | 5.12 | 4.84 | 4.90 | 4.70 | 4.74 | 6.00 |
| Cytochrome *c* | 6.20 | 6.00 | 5.10 | 5.84 | 6.00 | 6.10 | – | 5.40 | 6.00 | 5.14 | 5.26 | 5.58 |

the cation ($Mg^{2+}$, $Na^{+}$) at given anion and constant concentration; the valence of anion ($SO_4^{2-}$, $Cl^{-}$) at given cation and constant ionic strength; the type of cation and anion at constant ionic strength (0.02 *m* LiCl, 0.02 *m* NaCl, 0.02 *m* KCl, 0.02 *m* CsCl, 0.0067 *m* $MgCl_2$; 0.02 *m* NaCl, 0.02 *m* $NaClO_4$, 0.0067 *m* $Na_2SO_4$; 0.08 *m* NaCl, 0.08 *m* $NaClO_4$, 0.08/3 *m* $Na_2SO_4$).

There appears to be no correlation between $V_e$ and the ionic radius of the electrolyte cation in the eluent sequence 0.02 *m* LiCl, 0.02 *m* NaCl, 0.02 *m* KCl, 0.02 *m* CsCl. When the concentration and the type of cation are constant (0.02 *m* NaCl, 0.02 *m* $NaNO_3$, 0.02 *m* $NaClO_4$), the variation of electrolyte anion results in small changes in $V_e$ for aldolase, albumen (bovine), chymotrypsinogen and myoglobin. The substitution of $Na^{+}$ by $Mg^{2+}$ at given anion and constant concentration (0.02 *m* NaCl, 0.02 *m* $MgCl_2$) causes a higher retention for most proteins in the fractionation range, except for chymotrypsinogen, even at constant ionic strength (0.02 *m* NaCl, 0.0067 *m* $MgCl_2$). At 0.02 *m* NaCl and 0.0067 *m* $Na_2SO_4$ changing the valence of the anion results in an increase, a decrease or no change in $V_e$, depending on the protein. At constant ionic strength and given anion, the type and valence of the cation appears to have no effect on $V_e$. This is not the case for a given cation, constant ionic strength but variable anion [0.02 *m* (0.08) NaCl, 0.02 *m* (0.08) $NaClO_4$, 0.0067 *m* (0.0267) $Na_2SO_4$] at two ionic strength levels.

Unfortunately, the system behaves in such a complex way that the observed deviations of $V_e$ cannot be treated on a rigorous physico-chemical basis. Furthermore, the effects are small compared to those caused by changing the pH of the eluent. It appears that no purely ionic effect is responsible for the changes in $V_e$.

*Diol-ligand effects*

Owing to its chemical structure, the bonded ligand has a hydrophilic as well as a hydrophobic character. It therefore appears questionable to determine the effect of the diol group on the retention of proteins alone. A possible way to estimate the relative magnitude of the two structural effects could be to synthesize and test a series of diol-modified silicas which differ in the length of the *n*-alkyl group at a given terminal position and in the number of OH groups per ligand at constant *n*-alkyl chain length. Such an examination, however, is extremely tedious and again harbours several sources of error when it comes to comparing the results.

The primary question is how great is the effect caused by the ligand in relation to size exclusion and ionic interaction. This can be established quite simply by a controlled change of the solvent strength of the eluent, *e.g.*, by addition of methanol to the buffer. Table IV presents the results of such a study. A small but distinct decrease in $V_e$ of proteins is observed when the methanol content is increased stepwise from zero to 5% (v/v), with the exception of chymotrypsinogen. This decrease can be attributed to hydrophobic interactions arising from the hydrocarbon spacer groups. The opposite effect observed in the case of chymotrypsinogen indicates that the hydrophilic character of the diol group is dominant. The decreasing tendency of $V_e$ is also observed for the small-size ionic solutes. However, the $V_e$ of dextrans remains constant, in contrast to all other solutes. When comparing the elution volumes of proteins and dextrans of similar molecular weight, *e.g.*, albumen (egg) and dextran 40, the proteins are found to be retained more strongly. Hydrophobic interactions may also become dominant when the ionic interactions are suppressed, *i.e.*, at high ionic strength of the eluent, $\mu \gg 0.2$.

TABLE IV

ELUTION VOLUME ($V_e$ IN ml) OF PROTEINS, DEXTRANS AND IONIC SOLUTES ON A LICHROSORB® DIOL COLUMN (250 × 6 mm) WHEN METHANOL IS ADDED TO THE ELUENT

| *Solute* | *Methanol (v/v) Sörensen phosphate buffer, pH 7.4* | | | | *Methanol (v/v) Sörensen phosphate buffer, pH 7.4, +0.02 m NaCl* | |
|---|---|---|---|---|---|---|
| | *0* | *1* | *3* | *5* | *0* | *5* |
| Ferritin | 3.24 | 3.25 | 3.23 | 3.22 | 3.33 | 3.20 |
| Catalase | 3.38 | 3.34 | 3.34 | 3.30 | 3.40 | 3.30 |
| Aldolase | 3.46 | 3.44 | 3.42 | 3.38 | 3.44 | 3.38 |
| Albumen (bovine) | 3.48 | 3.48 | 3.46 | 3.38 | 3.56 | 3.48 |
| Albumen (egg) | 3.64 | 3.62 | 3.60 | 3.52 | 3.70 | 3.62 |
| Chymotrypsinogen | 5.74 | 5.80 | 5.80 | 5.78 | 5.30 | 5.43 |
| Myoglobin | 5.36 | 5.29 | 5.26 | 5.13 | 5.12 | 5.08 |
| Sulphanilic acid | 5.54 | 5.50 | 5.46 | 5.34 | 5.60 | 5.50 |
| Phenylalanine | 6.42 | 6.38 | 6.32 | 6.20 | 6.34 | 6.22 |
| Benzoic acid | 5.92 | 5.84 | 5.76 | 5.60 | 5.98 | 5.78 |
| Dextran 500 | 3.25 | 3.26 | 3.24 | 3.20 | 3.23 | 3.24 |
| Dextran 90 | 3.26 | 3.28 | 3.28 | 3.26 | 3.28 | 3.24 |
| Dextran 40 | 3.46 | 3.40 | 3.50 | 3.45 | 3.50 | 3.45 |
| Dextran 9.4 | 5.40 | 5.38 | 5.38 | 5.40 | 5.40 | 5.40 |

In conclusion, the diol ligand appears to have a minor but measurable effect on the elution behaviour of proteins and thus can be used for improving the selectivity.

*Column efficiency and column lifetime*

Unlike synthetic polymers[12], the pure proteins are monodisperse and therefore a relationship between the degree of polydispersity of the solute and the plate height does not exist, as is the case with the former. However, the plate height of the solute is still a function of the MW of the solute due to the increase of the diffusion coefficient with MW. For proteins of MW between 10,000 and 50,000 daltons, about 6000 theoretical plates per 250-mm column length could be achieved at optimum flow-rate. In addition to plate number, the peak symmetry of proteins is an important feature in separation. It was found that a change in the eluent composition dramatically affected the peak shape. For instance, peak symmetry was considerably improved at pH 8.0 compared to lower pH values. This may be due to the fact that the surface is covered more homogeneously with negative charges. A similar trend was observed when changing from high to low ionic strength. Peak efficiency and symmetry as well as recovery of proteins was found to be dependent on the type of electrolyte cation. For instance, $K^+$ provided the most unfavourable results. However, the results are too fragmentary for a complete understanding. Nevertheless, they should be taken into account when optimizing a separation.

The lifetime of diol-modified silica columns, in particular LiChrosorb Diol, was found to be excellent; long-term stability using the same eluent, stability upon

frequent change of eluent composition and reproducibility of $V_e$ when returning to the original eluent system (better than 2%) were exhibited. Prior to prolonged storage or when not in use, the buffer should be purged from the column first with water and thereafter by *n*-propanol. Saturation of the eluent with monosilicic acid was not necessarily required. For the examinations in this study the same LiChrosorb Diol column was used throughout without any noticeable changes in selectivity and performance, even with frequent alteration of eluent composition and using buffer up to pH 9.5.

## CONCLUSIONS

It was demonstrated that:

(i) the retention of proteins on diol-modified silica is based predominantly on a size exclusion mechanism;

(ii) ionic interactions contribute to retention mainly through the variation of eluent pH;

(iii) to some extent the diol-ligands also affect retention, depending on the solvent strength of the eluent.

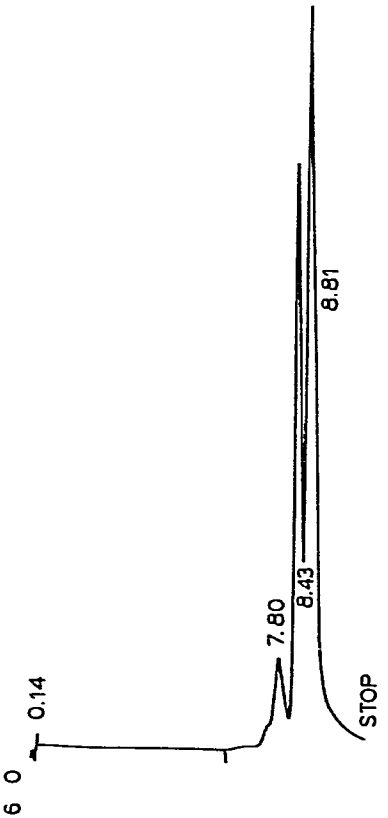


Fig. 3. Separation of two proteins, albumen (human) of MW 65,000 and transferrin of 80,000 daltons, on diol-modified silica (mean pore diameter 30 nm, $d_p$ = 5 μm). Column: 250 × 6 mm. Eluent: phosphate buffer (0.0645 *m* $Na_2HPO_4$, 0.002 *m* $KH_2PO_4$), pH 8.0.

Although the retention of proteins on diol-modified silicas has been treated here in a phenomenological rather than a profoundly theoretical manner, the various operating methods have provided in some, but not all, cases a higher resolution than might have been expected for a pure size exclusion mechanism. An example is given in Fig. 3, which shows a separation of two proteins having MW 65,000 [albumen (human)] and 80,000 (transferrin) daltons. Another advantage of this mode of HPLC is that it can readily be scaled-up for semi-preparative isolation of biopolymers.

ACKNOWLEDGEMENT

We gratefully acknowledge the assistance of Dr. von Steldern of the Institut für Immunologie, Johannes Gutenberg-Universität, Mainz.

REFERENCES

1 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1–25.
2 E. Pfankoch, K. C. Lu, F. E. Regnier and H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 430–441.
3 W. W. Yaw, J. J. Kirkland and D. D. Bly, *Modern Size Exclusion Chromatography*, Wiley-Interscience, New York, 1979.
4 P. Roumeliotis, K. K. Unger, J. Kinkel, G. Brunner, R. Wieser and G. Tschantz, in F. Lottspeich, A. Henschen and K. Hupe (Editors), *High Pressure Liquid Chromatography in Protein and Peptide Chemistry*, W. de Gruyter, Berlin, in press.
5 N. Becker and K. K. Unger, *Z. Anal. Chem.*, 304 (1980) 374–381.
6 D. E. Schmidt, R. W. Giese, D. Conron and B. L. Karger, *Anal. Chem.*, 52 (1980) 177–182.
7 N. Becker and K. Unger, *Chromatographia*, 12 (1979) 539.
8 P. Roumeliotis and K. K. Unger, *J. Chromatogr.*, 185 (1979) 445.
9 K. Unger, *Porous Silica (J. Chromatogr. Library*, Vol. 15), Elsevier, Amsterdam, 1979.
10 J. H. Knox, R. Kaliszan and G. J. Kennedy, *Proc. Faraday Symp. No. 15, Brighton, Dec. 1980*, in press.
11 D. Kerner and W. Leiner, *Colloid Polym. Sci.*, 253 (1975) 960–968.
12 J. H. Knox and F. McLennan, *Chromatographia*, 10 (1977) 75.