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# HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY ON ETHER-BONDED PHASES

## CHROMATOGRAPHIC CHARACTERISTICS AND GRADIENT OPTIMIZATION

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SUMMARY

This paper is a continuation of the evaluation of hydrophilic ether columns for the separation of proteins by hydrophobic-interaction chromatography. In this approach, linear salt gradients of decreasing concentration of ammonium sulfate yield sharp chromatographic peaks with high mass recovery and maintenance of biological activity. Mild adsorption conditions are indicated by the minimal changes in chromatographic peak area of native proteins as a function of contact time by the biopolymer with the stationary phase. Further evidence of minimal kinetic processes under the chromatographic conditions are seen in the constancy of isocratic retention with mobile phase flow-rate and sample load up to 2 mg. Based on the well-behaved chromatographic characteristics, we have explored gradient optimization in terms of the Snyder model for gradient elution. It is shown that changes in retention, peak capacity and peak height follow predicted gradient time dependencies. Moreover, the influence of particle diameter and column length are found to be in agreement with expected behavior, based on the model. As a consequence of the agreement, prediction of conditions for optimum separation for a particular problem are possible. Other studies examine the influence of specific ion effects, *e.g.*, $Mg^{2+}$ binding to protein, which can override retention based on predicted surface tension behavior. Some characteristics of the ether column for hydrophobic-interaction chromatography are shown, *e.g.*, column stability at pH 8 and sample capacity.

## INTRODUCTION

In a previous paper we reported on the use of ether bonded phases for high-performance hydrophobic-interaction chromatography (HIC)[1]. The mobile phase consisted of a linear decreasing salt gradient in a salting-out agent such as ammonium sulfate at pH 6. Such mild conditions permitted recovery of proteins in biologically active forms, and this was critically demonstrated in the purification of rat liver enzymes[2]. This may be contrasted with reversed-phase liquid chromatography

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(RPLC) with *n*-alkyl-bonded phases where loss in biological function is frequently observed[3].

HIC in the high-performance mode is a relatively recent addition to the rapidly advancing field of high-performance liquid chromatographic (HPLC) separation of biopolymers. The method is based on earlier work with agarose gels[4,5]. Several papers have appeared on HIC with small-particle-diameter silica-based packings[6–8] and gel materials[9–11].

This paper continues our examination of ether-bonded phases for HIC. We first demonstrate that the kinetic processes occurring on the *n*-alkyl bonded phases in RPLC[3] are substantially reduced on the ether columns. This leads to the possibility of using isocratic measurements to obtain gradient parameters for optimization. We employ a model of gradient elution developed by Snyder *et al.*[12] and demonstrate the "well-behaved" character of standard proteins on the ether phases. The model can subsequently be used to predict the influence of column length on chromatographic separation. These studies are followed by an examination of the importance of the selection of the salt for control of retention in HIC, and the specific ion effect of $Mg^{2+}$. Further experiments are presented on the stability of the ether phase at pH 8. Finally, some examples of the HIC elution of enzymes and $tRNA^{Phe}$ are shown. Taken together, these results demonstrate that HIC is a powerful mode for rapid separation of active biopolymers.

## EXPERIMENTAL

### *Equipment*

The gradient liquid chromatograph consisted of two Model M6000A solvent delivery pumps controlled by an M660 solvent programmer (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.), a Model SF770 variable-wavelength UV detector (used at 280 nm) (Kratos Analytical Instruments, Westwood, NJ, U.S.A.) and a Model 355 recorder (Linear Instruments, Reno, NV, U.S.A.). The chromatographic data were processed (*i.e.*, moment analysis, etc.) with a Nelson Analytical (Cupertino, CA, U.S.A.) Model 2600 Chromatography software package used in conjunction with an IBM (Baca Raton, FL, U.S.A.) Personal computer XT. The chromatographic system also included a Model NBE water bath, Haake-Büchler Instruments (Saddle Brook, NJ, U.S.A.).

The ether column containing the ether-bonded silica-based stationary phase II described in a previous paper[1] (column dimensions 100 × 4.6 mm I.D.) was prepared as described before and used with a precolumn (60 × 4.6 mm I.D.) containing the same bonded phase. A gradient delay volume of 5.1 ml was measured and subtracted from all chromatographic data presented. In order to prevent oxidation of the bonded ether ligands, oxygen was excluded from the system by the use of stainless-steel tubing and continuous sparging of the mobile phases with helium.

### *Chemicals*

HPLC-grade water, organic solvents and reagent-grade sodium sulfate were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.) or Anachemia Chemicals (Champlain, NY, U.S.A.). Ammonium acetate and Grade III ammonium sulfate, as well as various high-quality protein standards, were obtained from Sigma (St. Louis,

MO, U.S.A.) and used as received. A sample of yeast $tRNA^{Phe}$ was a gift from Professor Rainer Bischoff (Max Planck Institut für Experimentelle Medizin, Göttingen, F.R.G.).

*Chromatographic procedures*

Mobile phases were prepared by adding the correct weight of salt and buffer to a volumetric flask containing HPLC-grade water previously degassed by aspirator vacuum. The pH was adjusted to 6.0 or the appropriate value with either glacial acetic acid or ammonium hydroxide, and a small amount of HPLC-grade water (degassed) was added to the mark. Solutions with high salt concentrations were not allowed to remain in the column or pumps for long periods of time. Unless otherwise indicated, standard mobile-phase conditions were employed, consisting of 3 *M* ammonium sulfate, 0.5 *M* ammonium acetate (pH 6.0) as mobile phase A and 0.5 *M* ammonium acetate (pH 6.0) as mobile phase B. A standard 20-min linear gradient (0 to 100% B) was used in all experiments with a flow-rate of 1.0 ml/min at 25°C. Exceptions to these conditions are noted in the paper. Protein solutions (5–10 mg/ml) were freshly made up in water. When not in use, the samples were stored at −20°C.

## RESULTS AND DISCUSSION

*Surface kinetic behavior*

We have previously shown[3,13] that in reversed-phase liquid chromatography on *n*-alkyl-bonded phases, conformational unfolding of proteins on the bonded phase surface can occur. Hearn and Grego[14] have also observed this behavior. The unfolding is a consequence of the high interfacial tension existing between the mobile phase and the bonded stationary phase[15]. Our previous work has strongly suggested that the reduction in the interfacial tension could significantly reduce this dynamic process. Studies in HIC on the ether column have already indicated that labile proteins can be recovered in an active state[2], strongly suggesting that the adsorption process is mild. We decided to test this point further.

Table I shows the results of on-column incubation for two standard proteins,

TABLE I

EFFECT OF ON-COLUMN INCUBATION ON THE RECOVERY OF PROTEIN IN HIC ON THE ETHER COLUMN

Conditions: isocratic hold at 3 *M* ammonium sulfate, 0.5 *M* ammonium acetate (pH 6.0). Gradient: see Experimental section.

| *Incubation time (min)* | *Peak area (%)** | |
|---|---|---|
| | *RNase A* | *α-CHTG* |
| 0 | 100 | 100 |
| 5 | 97.2 | 100 |
| 30 | 95.0 | 93.2 |
| 55 | 95.1 | 95.0 |
| 120 | 95.6 | 88.0 |

* R.S.D. = 2%.

ribonuclease A (RNase A) and α-chymotrypsinogen (α-CHTG). In this experiment, an aqueous solution of the protein was injected into the column containing a mobile phase of 3 *M* ammonium sulfate, and an isocratic hold was maintained for a period of time, *i.e.*, the incubation time. Subsequent to this, the normal linear gradient was begun. When a zero incubation time (*i.e.*, no isocratic hold) was used as the standard, it can be seen from the table that the peak area for RNase A does not change to any significant extent over a 2-h incubation time. In the case of α-CTHG, there is a 12% drop in the 2-h period; however, this is far less than previously found for this and several other globular proteins in RPLC[3].

We have previously proposed two processes of denaturation in RPLC: first, a rapid denaturation when the protein contacts the *n*-alkyl-bonded phase and, second, a slower unfolding for the protein remaining on the bonded phase surface. The results of Table I suggest that in HIC on the ether phase the rapid denaturation step is not significant and that if occurring, the slow denaturation step has a significantly greater half-life than found for RPLC. It is possible that a small amount of α-CHTG has altered its conformation on the surface, causing a loss in peak area. Clearly, however, other causes for the small loss are possible. Nevertheless, the conclusion of our previous work in RPLC that the contact time of the protein with the surface be kept to a minimum remains true for HIC as well. The stability of the native state on the ether phase is a result of the greater hydrophilicity of this phase, relative to the *n*-alkyl phase, resulting in a lower interfacial tension between the mobile and stationary phases in HIC.

The mildness of the adsorption process can be further seen in Table II, where the retention of RNase A under isocratic conditions as a function of mobile phase flow-rate is presented. In a previous study on RPLC[16], it has been shown that isocratic retention was a strong function of flow-rate for biopolymers. However, with

TABLE II

HIC ISOCRATIC RETENTION DATA FOR RNase A AS A FUNCTION OF FLOW-RATE ON THE ETHER COLUMN

Mobile phase: 1.9 *M* ammonium sulfate, 0.5 *M* ammonium acetate, pH 6.0. Other conditions: see Experimental section.

| *F (ml/min)* | *k'* |
|---|---|
| 0.20 | 2.20 |
| 0.30 | 2.40 |
| 0.40 | 2.32 |
| 0.50 | 2.41 |
| 1.0 | 2.56 |
| 1.5 | 2.43 |
| 1.9 | 2.42 |
| 2.4 | 2.51 |
| 2.8 | 2.50 |
| 3.5 | 2.54 |
| 4.0 | 2.20 |
| Average: | 2.4 ± 0.13 |

HIC on the ether phase, it can be seen in the table that the capacity factor remains essentially constant with a 20-fold change in flow-rate. The results in Table II are further supported by the fact that retention in the isocratic mode was independent of sample size over the range from 77 $\mu$g to 2 mg, the highest value examined for both RNase and $\alpha$-CHTG. (Retention was measured on the basis of the first statistical moment of the peak, and it is worth noting that the asymmetry factor for isocratic elution was roughly 1.2.) Previously, we[17] have found in RPLC that retention was strongly sample size-dependent in the isocratic mode. The results of Table II are further evidence of the mild adsorption behavior of the HIC system under study.

Based on this behavior, we next determined in the isocratic mode the $S$ values for the standard proteins, *i.e.*, the slopes of the plots of log $k'$ *vs.* concentration fraction of ammonium sulfate (1 = 3 m$M$ ammonium sulfate, 0 = 0 m$M$ ammonium sulfate), other conditions being maintained constant, as described in Experimental. The plots were found to be linear, yielding an $S$ value for RNase A of 7.9 ($r$ = 0.9982) and that of $\alpha$-CHTG, 6.4 ($r$ = 0.9972). The isocratically determined $S$ values for these proteins agree well with those previously found by use of gradient elution[1]. Thus, in this system, isocratic data can be directly transferred to gradient elution.

*Gradient optimization*

From the above discussion it is clear that a linear gradient of decreasing ammonium sulfate concentration will yield a linear change in log $k'$ per unit time, *i.e.*, linear solvent strength (LSS) conditions[18]. When LSS conditions are employed, the relationships of retention, peak capacity and peak height to chromatographically important parameters can be greatly simplified, leading to an understanding and a prediction of the effects of these parameters on separation and analysis.

In this work, we have followed the model of Snyder and co-workers[12,16,18,19], for LSS gradient elution. A basic expression relating retention to gradient conditions with LSS gradient is shown in eqn. 1:

$$\log k'_i = \log k'_0 - b\,(t/t_0) \tag{1}$$

where $k'_i$ is the capacity factor at the column inlet at time $t$, $k'_0$ is the capacity factor at the start of the gradient, and $t$ and $t_0$ represent the time of the gradient and the time of an inert peak, respectively. The gradient steepness parameter, $b$, is the rate of change of elution strength of the mobile phase per unit time and can be expressed by

$$b = \frac{\varphi S V_m}{F t_G} \tag{2}$$

where $\varphi$ is the fraction of the strong or B solvent used in the gradient, $t_G$ is the time of the gradient, $V_m$ the mobile-phase volume and $F$ the mobile-phase volumetric flow-rate.

The $b$ value is inversely proportional to the capacity factor of the solute. Since in gradient elution $k'$ varies from point to point in the column, it is convenient to

select $\bar{k}'$, the $k'$ value of the solute when it passes the midpoint of the column. The relationship between $b$ and $\bar{k}'$ can be written as

$$b = \frac{1}{1.15\bar{k}'} \tag{3}$$

From eqns. 2 and 3, it can be seen that large values of $t_G$ yield large capacity factors, whereas large $S$ values, other things being equal, yield small capacity factors.

The gradient retention time ($t_g$) of the solute can be written as follows,

$$t_g = \frac{t_G}{\varphi S} \log (2.3bk'_0 + 1) + t_0 \tag{4}$$

In eqn. 4 the delay time has been subtracted out. As shown previously[1], the extent of size exclusion from the porous matrix is small for the proteins studied in this work. Accordingly, for simplicity, we have neglected the size-exclusion correction to retention[16]. The value of $k'_0$, as measured from the isocratic retention results for RNase A, was found to be $2 \cdot 10^2$, whereas that for α-CHTG was $10^4$. In this work, the $b$ value is in general roughly 0.3, and as a consequence, we can simplify eqn. 4 by noting the following approximations:

$$2.3bk'_0 \gg 1 \tag{5}$$

and

$$\log k'_0 \gg | \log 2.3b | \tag{6}$$

which yields

$$t_g = \frac{t_G}{\varphi S} \log k'_0 + t_0 \tag{7}$$

Thus, $t_g$ should be proportional to $t_G$, since the second term, $t_0$, is constant. A plot of $t_g$ *vs.* $t_G$ is shown in Fig. 1A, for three standard proteins: RNase A, ovalbumin (OVA) and α-CHTG. The $t_g$ value is seen to be directly proportional to the time of the gradient. Note that other parameters, such as $S$, $\varphi$ and $V_m/F$ are maintained constant.

We next consider the resolving power of the column under given gradient conditions. This resolving power can be most easily expressed as the peak capacity, PC, which can be defined as in eqn. 8

$$\mathrm{PC} = \frac{t_G}{4\sigma_t} \tag{8}$$

where $\sigma_t$ is the time based standard deviation, averaged over the protein peaks in the gradient. $\sigma_t$ can be obtained by the square root of the second centralized moment. For the measurement of the second centralized moment, we used the recently developed empirical equation based on the width at half height[20]. Typical chromatograms can be observed in Figs 2 and 3. In this analysis, we focused only on RNase A and

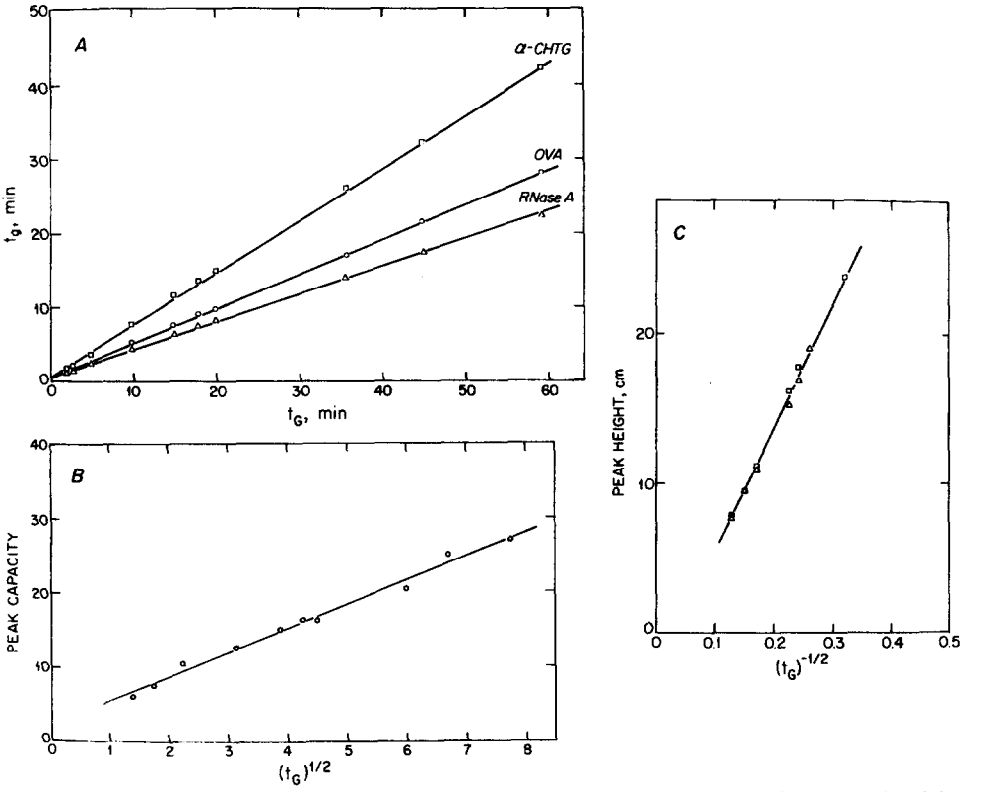


Fig. 1. Influence of gradient time, $t_G$ on (A) protein retention time, $t_g$; (B) peak capacity; (C) peak height under HIC gradient elution conditions. All conditions except gradient time are listed in the Experimental section. Protein retention time is corrected by subtraction of the system delay time (5.1 min). OVA = Ovalbumin. Identification in C: △ = RNase A, □ = α-CHTG.

α-CHTG. Since cytochrome *c* (CYT) is eluted at the very beginning of the gradient, it was not included in the determination of $\sigma_t$. In addition, the enhanced broadening of OVA with sample age, noted previously[1], suggested that this protein be omitted (see discussion at the end of this section).

Snyder *et al.*[12] have previously shown that peak capacity is proportional to $(t_G)^{1/2}$. However, it is instructive to derive the full expression of this relationship in order to understand the importance of various terms to peak capacity. This derivation can be easily performed by solving for the time-based standard deviation $\sigma_t$. Eqn. 9 is an expression for $\sigma_t$[18].

$$\sigma_t = \frac{(1 + k')V_m}{2.3b\, k'F\,(N)^{1/2}} \tag{9}$$

where $N$ represents the number of theoretical plates in the column. Since diffusion coefficients $D_m$, of proteins are more than an order of magnitude less than those for small molecules (*i.e.*, for proteins $D_m \approx 10^{-6}$ cm²/sec), high reduced velocities $v$ are found for the large molecules at the flow-rates commonly used for small molecules.

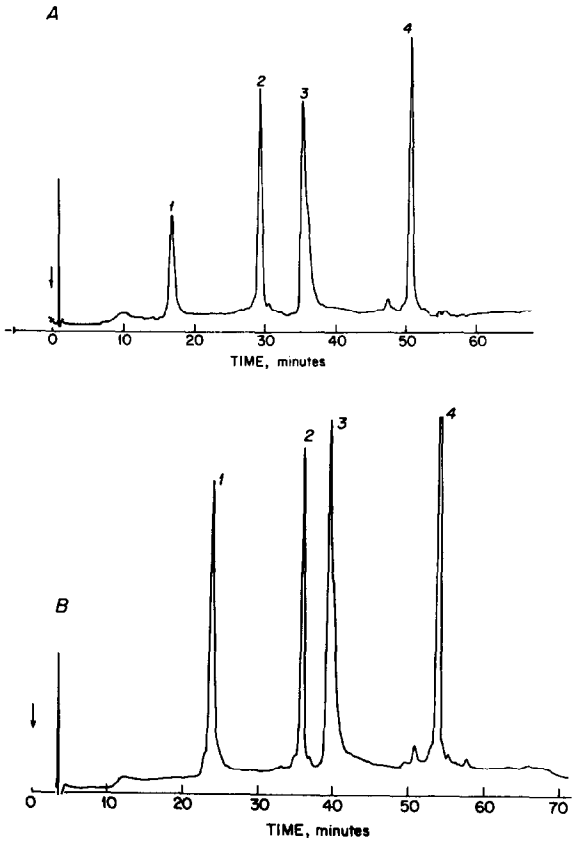


Fig. 2. (A) HIC separation of standard protein mixture on the ether column. Conditions: see Experimental section, except $t_G$ = 60 min. Standard proteins: 1 = cytochrome *c*, 50 μg injected; 2 = RNase A, 150 μg; 3 = OVA, 200 μg; 4 = α-CHTG, 50 μg. (B) HIC separation of standard protein mixture on the ether column. Conditions as in A, except that a 30-cm column length (*i.e.*, three 10-cm columns coupled in series) was employed.

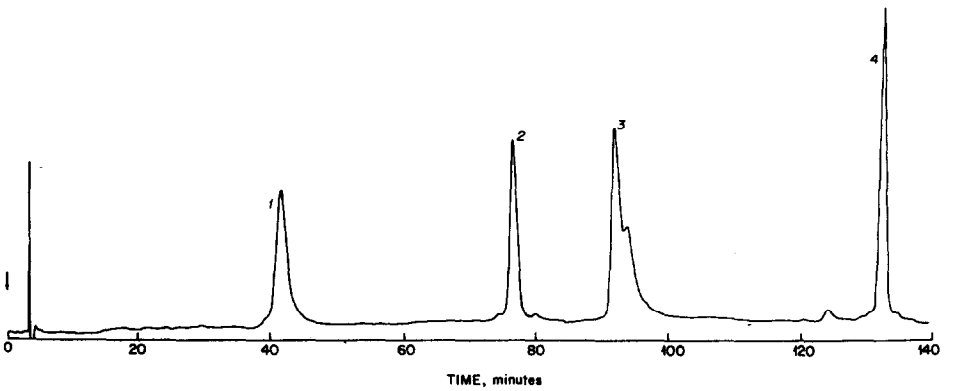


Fig. 3. HIC separation of standard protein mixture on the ether column. Conditions as in Fig. 2B, except $t_G$ = 180 min.

Indeed, in our system, the reduced velocities are generally greater than 100. Under such circumstances, the reduced plate height-reduced velocity equation can be written as

$$h = Cv \tag{10}$$

where $h$ = reduced plate height and $C$ = proportionality constant. From the well-known definitions of $h$ and $v$, eqn. 10 can be converted into an expression related to $N$, the theoretical plates, as

$$N = \frac{D_m t_0}{C d_p^2} \tag{11}$$

From the fact that $V_m/F = t_0$, and by substitution of $b$ from eqn. 3, eqn. 2 can be converted to

$$t_0 = \frac{t_G}{1.15k' \varphi S} \tag{12}$$

Eqn. 12 can be substituted into eqn. 11 to yield

$$N = \frac{t_G D_m}{1.15k' \varphi S C d_p^2} \tag{13}$$

This expression for $N$ can be inserted into eqn. 9, followed by the substitution into eqn. 8 to give the final expression for PC

$$\mathrm{PC} = 0.5 (k')^{-0.25} C^{-0.5} d_p^{-1} D_m^{0.5} (t_G \varphi S)^{0.5} \tag{14}$$

In this equation we, like Snyder *et al.*[12], have used the approximation, that $[\bar{k}'/(1 + \bar{k}')] \approx (\bar{k}')^{0.25}$.

Next consider peak height. The peak height, PH, normalized to the peak area $A_v$ in volume units, can be written as

$$\frac{\mathrm{PH}}{A_v} = \frac{1}{F\sigma_t\sqrt{2\pi}} \tag{15}$$

Substitution for $\sigma_t$ in a manner similar to the above yields

$$\frac{\mathrm{PH}}{A_v} = 0.86 (k')^{-0.25} C^{-0.5} d_p^{-1} D_m^{0.5} F^{-1} \left(\frac{S\varphi}{t_G}\right)^{0.5} \tag{16}$$

Eqns. 14 and 16 reveal a low dependence of peak capacity and peak height on the mean capacity factor $\bar{k}'$. The capacity factor dependence is probably moderated even further by the dependence of $C$ on the capacity factor[12]. However, since this dependence has not been directly measured for HIC on the ether column, we omit this point. Nevertheless, it is fair to say that because of the low dependence on capacity factor, other terms in eqns. 14 and 16 control the changes in value of PC and PH.

Fig. 1B shows a plot of peak capacity *versus* the square root of the gradient time, $t_G$. It can be observed that a direct linear relationship exists between PC and $(t_G)^{1/2}$, as predicted in eqn. 14. Note that the elution time $t_g$ increases linearly with gradient time (Fig. 1A), whereas the resolution or peak capacity increases with the square root of $t_G$. In this sense, the gradient time is analogous to column length in isocratic operation. Fig. 1C shows a plot of normalized peak height for RNase A and α-CHTG *vs.* $(t_G)^{1/2}$. In agreement with the prediction of the model, a linear relationship is again found. Thus, longer gradient times improve resolution at the expense of peak height and analysis time. Knowledge of these dependencies permits the selection of appropriate trade-offs.

Eqns. 14 and 16 predict that peak capacity and peak height will both be inversely proportional to particle diameter. We have confirmed this prediction by examining particle diameters of 4, 6 and 7 μm, all composed of the ether bonded phase. Under the same gradient conditions, the peak capacity varies from 18 at 7 μm to 22 at 6 μm to 32 at 4 μm. These values are directly proportional to the inverse of the particle diameter. The relative peak height varies from 4.5 at 7 μm to 5.3 at 6 μm to 7.8 at 4 μm, again an inverse relationship to particle diameter. Moreover, as predicted in eqn. 4 and observed, the elution time does not vary with the change in particle diameter. The relationships of PC and pH to $d_p$ reveal that the *h vs. v* plot is independent of particle diameter in this case, or in other words that the columns are equivalently packed. Thus, a decrease in $d_p$ for HIC can lead to improved PC and PH, with no change in analysis time.

Another parameter which can be examined, based on the above model, is column length. We compared a 10-cm to a 30-cm column, in which the 30-cm column consisted of three 10-cm columns connected in series. Table III shows the results of this study, in which the gradient time was varied from 20 to 180 min. Previous RPLC experiments have shown that roughly identical chromatograms are obtained with different column lengths, when the same gradient conditions are used[21,22]. Similar

TABLE III

INFLUENCE OF COLUMN LENGTH ON RESOLUTION IN THE GRADIENT ELUTION HIC SEPARATION OF PROTEINS

Chromatographic conditions: see Experimental section.

| $t_G$ (min) | $\bar{k}$* | $t_g$** (min) | PC*** | PH§ |
|---|---|---|---|---|
| *Column length, 10 cm* | | | | |
| 20 | 2.4 | 14.3 | 18 | 5.8 |
| 60 | 7.2 | 39.8 | 33 | 3.3 |
| *Column length, 30 cm*§§ | | | | |
| 20 | 0.81 | 17.4 | 17 | 6.7 |
| 60 | 2.4 | 43.8 | 32 | 3.5 |
| 180 | 7.2 | 116.3 | 54 | 1.8 |

* Capacity factor as solute passed column midpoint.
** Gradient retention time for α-CHTG, corrected for a gradient delay time of 5.1 min.
*** Peak capacity = $t_G/4\sigma_t$, where $\sigma_t$ is averaged for RNase A and α-CHTG.
§ Peak height for α-CHTG normalized for peak area.
§§ Three 10-cm columns coupled in series.

results were obtained in our work. Fig. 2 shows chromatograms of four standard proteins on the ether column with $t_G$ = 60 min for the 10- and 30-cm columns, respectively, and it is evident that the separation is roughly equivalent. Indeed, the values in Table III quantitatively indicate this to be the case.

One reason suggested for the apparent column length independence in RPLC has been the large $S$ values for proteins, which would imply possible displacement rather than elution development. However, in HIC with the ether column, we have already noted much smaller $S$ values, lower indeed by a factor of 6–7. As predicted earlier[12] and clearly seen in eqns. 14 and 16 and the data of Table III, the cause of the above column length results lies elsewhere. Eqns. 14 and 16 reveal no column length dependence of PC or PH if $t_G$ is maintained constant. (We assume again a negligible PC and PH dependence on $\bar{k}'$.) Qualitatively, the increase in theoretical plates on the longer column is counterbalanced by the lower $\bar{k}'$ value on this column with constant $t_G$ (see Table III), assuming $\bar{k}'$ is not too large, *e.g.* 6 or less.

Consider next maintaining $\bar{k}'$ constant. This can be achieved on the 30-cm column by increasing $t_G$ by a factor of three (*i.e.* $t_G$ = 180 min). As seen in Table III, PC now increases up to 54, and PH is reduced to 1.8. The chromatogram for $t_G$ = 180 min is illustrated in Fig. 3. (Note the sharp peaks even for such a shallow gradient.) From this discussion, it can be concluded that the model can directly account for column length changes.

The excellent correspondence of the results in HIC on the ether column with the gradient model of Snyder is important in that rational approaches to optimization can be taken. However, as a caveat it must be added that the proteins must behave in an "ideal" fashion. The complexity of biopolymers is such that non-ideal phenomena, such as conformational changes, aggregation, subunit release, etc., on the stationary or in the mobile phase may occur in specific cases. These changes can lead to chromatographic behavior that overwhelms the sharp, symmetrical peaks found with simple, globular proteins. Thus, for example, we omitted OVA from this analysis because of the enhanced broadening potentially possible with aged samples. In summery, the model represents a benchmark upon which to base criteria for "ideality".

*Role of surface tension on retention*

In RPLC, a critical parameter controlling retention is the surface tension of the mobile phase. Based on the solvophobic theory used in RPLC, Melander and Horváth[23] have developed a set of retention equations for HIC in which the surface tension of the aqueous salt solution is again a critical parameter controlling retention. Salts significantly influence the surface tension of water, based on their type and concentration. Indeed, the Hofmeister series of salting out capability of various salts can be correlated with the molal surface tension increment of these salts[23].

The surface tension of the mobile phase was found to be an important parameter controlling HIC retention on the ether column as well. This can be seen in Fig. 4, which is a plot of the surface tension difference (relative to water) at the point of elution when ammonium sulfate and sodium sulfate are used as salts. The effect of a given salt on retention can thus be directly predicted.

The above surface tension correlation is only true in the absence of specific ion effects. As shown by others, with certain proteins the use of $Mg^{2+}$ or $Ca^{2+}$ can result in changes in protein solubility from that anticipated on the basis of the molal surface

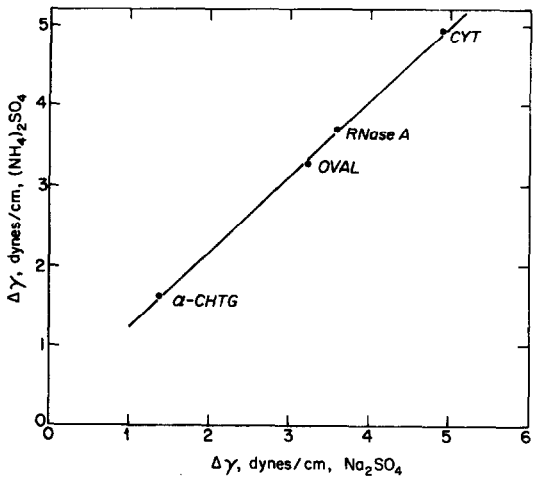


Fig. 4. Effect of surface tension on retention in HIC. $\Delta(\gamma)$ = Surface tension at elution point minus surface tension of water (72 dynes/cm). Conditions: ammonium sulfate salt: see Experimental section. For sodium sulfate as salt: mobile phase A = 2 *M* sodium sulfate, 0.5 *M* ammonium acetate at pH 6.0. All other conditions: see Experimental section.

tension increment[24]. We decided to examine this effect by measuring retention with 0.6 *M* magnesium chloride added to the B solvent. The results are shown in Table IV. It can be seen that for RNase A and α-CHTG, an increase in retention is observed when $Mg^{2+}$ is included in the B solvent, whereas no change is observed for α-lactalbumin (α-LACT), a known Ca/Mg-binding protein[25]. The results reveal that selectivity can be achieved by additives to the mobile phase for HIC with this system. Lindahl and Vogel[26] have used metal binding with α-LACT to manipulate retention in HIC on agarose columns. In Table IV we also calculated the expected retention of the three proteins, based on elution at an equivalent surface tension to that in the absence of $Mg^{2+}$. It can be seen that in all cases a lower experimental retention is found. In other words, the proteins are made more hydrophilic by the specific ion

TABLE IV

INFLUENCE OF MAGNESIUM ON RETENTION IN HIC

Gradient conditions: mobile phase A: 2 *M* ammonium sulfate + 0.5 *M* ammonium acetate, pH 6.0; mobile phase B: 0.5 *M* ammonium acetate, pH 6.0 or mobile phase B: 0.6 *M* magnesium chloride + 0.5 *M* ammonium acetate, pH 6.0. Other conditions: see Experimental section.

| *Protein* | $V_g^*$ *(ml)* | | |
|---|---|---|---|
| | *Without* $Mg^{2+}$ | *With* $Mg^{2+}$ | *Calc.*** |
| RNase A | 1.84 | 2.65 | 3.2 |
| α-LACT | 8.62 | 8.62 | 15.0 |
| α-CHTG | 13.16 | 15.31 | 23.2 |

* Gradient retention volume corrected for delay volume.

** Calculated protein retention volume for $Mg^{2+}$ added to solvent B, based on equivalent surface tension as for elution without $Mg^{2+}$ in solvent B.

effect. The greatest difference is that due to α-LACT, as expected. At a later date we shall report further on the use of additives to the mobile phase in HIC to influence retention.

*Chromatographic characteristics*

In a previous paper[1], it was shown that the ether column was stable for at least five months of continued use at pH 6, based on HIC and highly sensitive size-exclusion chromatographic (SEC) measurements at low ionic strength. In general, silica-based hydrophilic bonded phases are considered relatively unstable above pH 7, particularly with high salt concentrations in the mobile phase. However, the capability of operating at pH 7–8 would clearly be useful in HIC, given that physiological pH is 7.4. Accordingly, we decided to examine the ether column stability at pH 8.

Table V shows the HIC retention of RNase A, OVA and α-CHTG after roughly 5, 10 and 15 l were used at pH 8, and 25°C. As can be seen, the gradient retention volume $V_g$ for these proteins remains constant with an R.S.D. of 1–3%, which is approximately the same R.S.D. as obtained at pH 6[1]. The higher R.S.D. for OVA found here, and previously at pH 6, may be related to the fact that the $V_g$ of the species is very sensitive to the time the protein spends in solution[1]. It is also interesting to note that the $V_g$ values at pH 6 and 8 are identical, within experimental error (data not shown). A survey of a number of other proteins agrees with this finding, namely that $V_g$ does not change between pH 6 to 8, unless the p*I* of the protein occurs in this region. Kato *et al.*[10] have also observed this result.

We also tested the stability of the columns at pH 8 in the SEC mode, using 0.50 *M* and 0.050 *M* ammonium acetate, both pH 6, as mobile phases. Leakage of bonded phase ligands from the surface may yield small negatively charged regions on the silica which would retard basic proteins such as lysozyme (LYS). Table VI shows the results of this study. Note that even after 15 l, lysozyme still does not have

TABLE V

STABILITY OF ETHER PHASE FOR PROTEIN RETENTION AT pH 8

Mobile phase: 0.5 *M* ammonium acetate, pH 8.0, at 25°C; flow-rate 1.0 ml/min. Other conditions: see Experimental section.

| *Volume of mobile phase passed (l)* | $V_g$* *(ml)* | | |
|---|---|---|---|
| | *RNase A* | *OVA* | *α-CHTG* |
| 0 | 8.3 | 9.5 | 14.1 |
| 5.3 | 8.3 | 9.2 | 14.1 |
| 10.5 | 8.4 | 9.6 | 14.1 |
| 15.7 | 8.5 | 10.0 | 14.4 |
| Mean | 8.4 | 9.6 | 14.2 |
| R.S.D. (%) | 1.1 | 3.5 | 1.1 |
| Mean, pH 6** | 8.7 | 10.4 | 14.5 |
| R.S.D. (%) | 2.0 | 5.0 | 2.2 |

* Protein elution volume corrected for gradient delay volume.

** From ref. 1.

TABLE VI

COLUMN STABILITY AT pH 8 AS MEASURED BY SEC RETENTION

$T$ = 25°C; $V_0$ = 1.2 ml.

| *Protein* | *$V_g$ (ml)* | | | | | |
|---|---|---|---|---|---|---|
| | *0.50 M ammonium acetate, pH 6.0* | | | *0.050 M ammonium acetate* | | |
| | *Volume of mobile phase passed (l)* | | | *Volume of mobile phase passed (l)* | | |
| | *0** | *5* | *15* | *0** | *5* | *15* |
| Bovine serum albumin | 0.88 | 0.87 | 0.88 | 0.85 | 0.85 | 0.88 |
| RNase A | 0.99 | 0.98 | 1.00 | 0.99 | 1.00 | 0.99 |
| α-CHTG | 0.99 | 0.98 | 1.00 | 0.98 | 1.00 | 1.02 |
| LYS | 1.0 | 1.0 | 1.10 | 1.09 | 1.10 | 1.20 |

* 0.5 *M* ammonium acetate, pH 8.0; $T$ = 25°C; flow-rate = 1.0 ml/min.

a retention value greater then $V_0$ (1.2 ml) with the 0.50 *M* buffer at pH 6.0. Thus, the ether column appears stable at pH 8 for long periods of time.

The passage of 15 l of mobile phase is roughly equivalent to one and a half months of continual use. It is likely that the column can be used in the HIC and SEC modes for significantly longer periods of time. Undoubtedly, the good stability is related to the fact that bonding was accomplished with a triethoxysilane. It should also be noted that, in order to achieve high stabilities with any column, care must be exercised with the type of samples injected and, when dirty samples are used, a guard column between the injector and chromatographic column must be employed.

One other chromatographic characteristic of interest is the sample capacity of the ether column in HIC. The assessment of this characteristic is difficult, since it obviously depends on the sample components, matrix, and concentration, as well as the method of characterization. Fig. 5 shows a plot of gradient retention volume

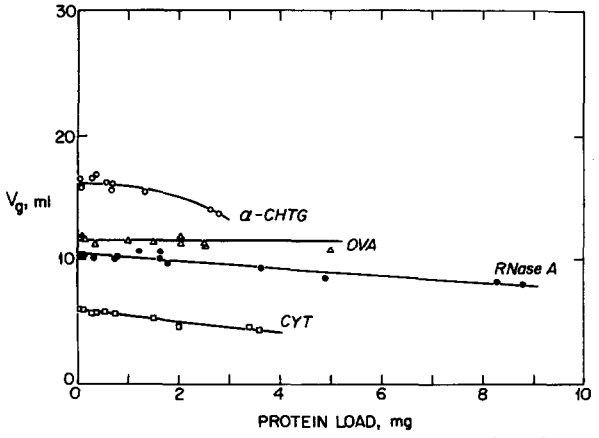


Fig. 5. Influence of protein sample size on retention volume in gradient HIC. Conditions: see Experimental section. All proteins were injected as a mixture. $V_g$ is corrected for delay volume, 5.1 ml.

(corrected for delay volume) as a function of mg of protein in a four-component standard aqueous mixture, the relative amounts of each being CYT:RNase A: OVA:α-CHTG = 1:3:4:1. As can be seen, mg amounts of protein can be injected into the 10 cm × 4.6 mm I.D. column before significant changes in $V_g$ occur. Indeed, because of the wide separation of the four peaks, we have successfully resolved 4–8 mg of each protein in one chromatogram. Thus, for well-defined samples, mg capacity is achievable on the short column.

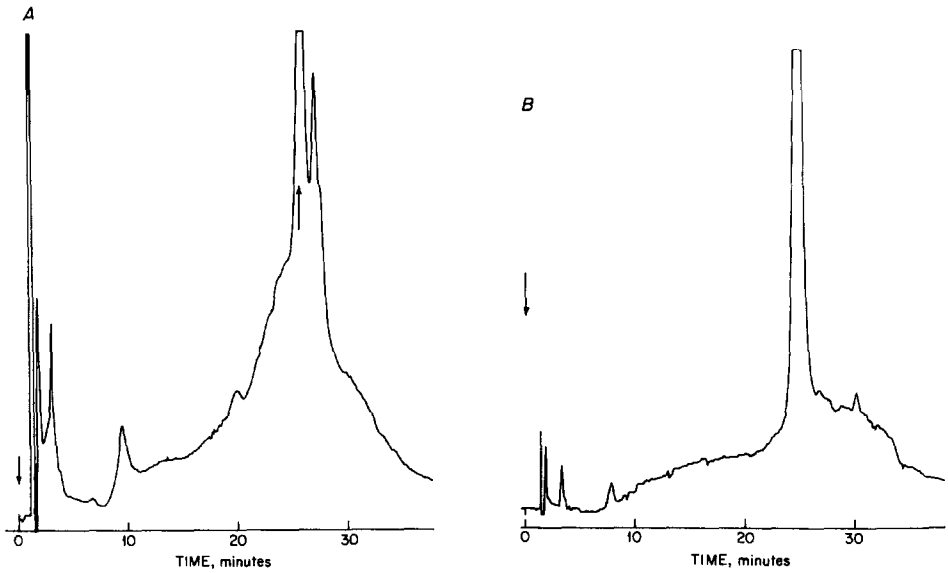


Fig. 6. HIC of β-amylase on the ether column. Chromatographic conditions: see Experimental section. A, β-amylase: specific activity, 15.7 units/mg; injection volume, 50 μl; amount, 250 μg. B, purified β-amylase: specific activity, 845 units/mg; injection volume, 5 μl; amount, 134 μg.

Let us next turn to several examples of biopolymer elution which further illustrate the capabilities of the ether column. These examples supplement the previous paper[1]. Fig. 6 shows the rapid separations of a crude and a fairly pure sample of β-amylase, and Fig. 7 shows the corresponding separations for crude and purified lipoxidase. HIC clearly appears to be useful in a rapid assessment of compound purity. Fig. 8 shows the elution of a relatively pure sample of t-RNA$^{Phe}$ from yeast. We have chromatographed a number of purified tRNA samples; HIC would appear to be capable of broad use in this application. Moreover, HIC is performed under conditions in which proteins are favored to remain in their native state. We have found this to be true in some of our own studies on the ether column. We have isolated in rapid fashion rat liver enzymes from mitochondrial extracts, with up to a 15-fold enhancement in specific activity[2]. Currently, we are examining cytochrome c oxidase, a multiple subunit integral membrane protein and have recovered this species from the ether column in an active state. The results of this latter work will be reported separately. HIC would appear to be suited to the purification of very hydrophobic and labile species.

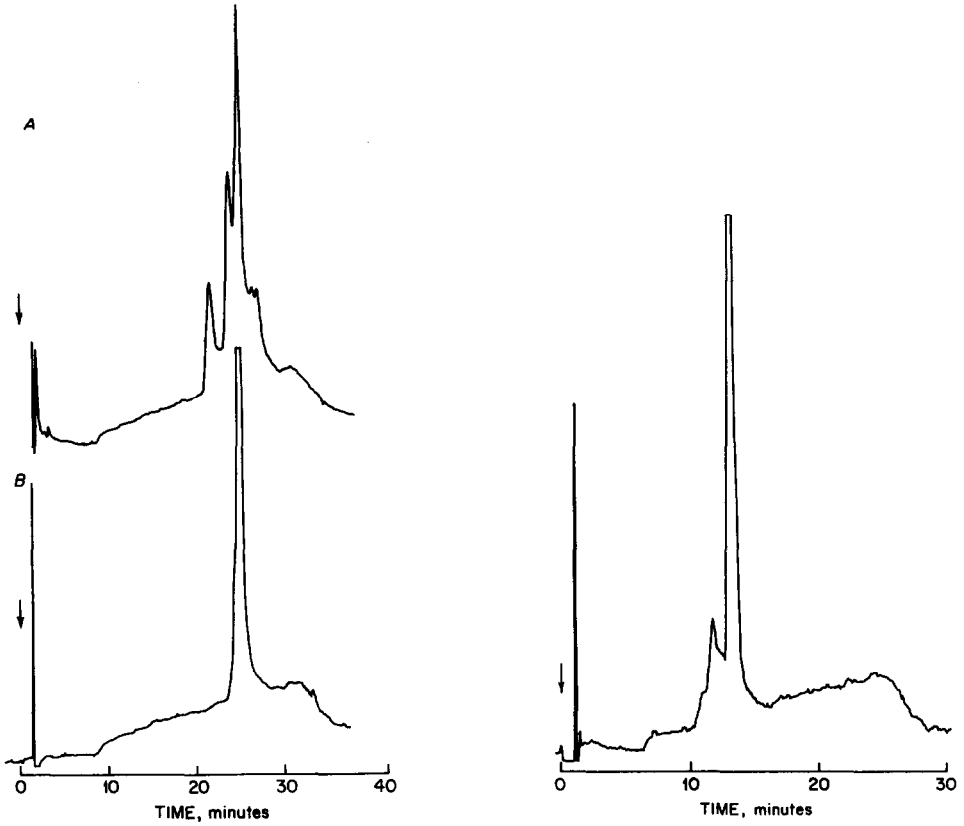


Fig. 7. HIC of lipoxidase on the ether column. Chromatographic conditions: see Experimental section. A, lipoxidase: specific activity, 300 K units/mg; injection volume, 12.5 μl; amount, 125 μg. B, purified lipoxidase: specific activity, 735 K units/mg; injection volume, 60 μl; amount, 120 μg.

Fig. 8. HIC of purified $tRNA^{Phe}$ on the ether column. Chromatographic conditions: see Experimental section, except detection at 260 nm 0.1 a.u.f.s. Injection volume, 3.5 μl; amount, 3.5 μg.

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