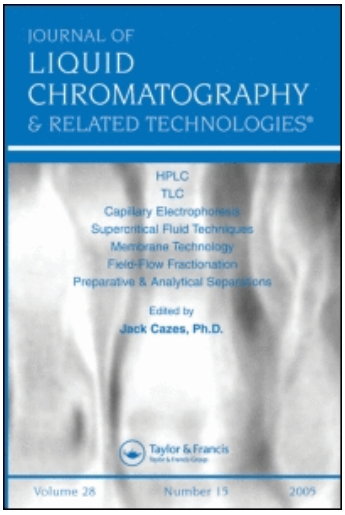

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Hydrophobic Interaction Chromatography of t-RNA's and Proteins

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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF t-RNA's AND PROTEINS

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ABSTRACT

Various microparticulate siliceous bonded stationary phases having weakly hydrophobic ligates were developed for HPLC of proteins and t-RNA's by hydrophobic interaction chromatography (HIC). It was confirmed that optimal separation of different types of biopolymers can be obtained by using a set of stationary phases having appropriate hydrophobic properties. Thus, the separation of t-RNA's is best carried out on stationary phases which are more hydrophobic than those optimal for HIC of proteins. Plots of log k' of both proteins and t-RNA's against the salt molality in the eluent yielded straight lines at sufficiently high salt concentrations in the eluent. The limiting slopes represent the hydrophobic interaction parameter for the particular chromatographic system and can serve as measures of the hydrophobic character of either the biopolymer or the stationary phase. Stationary phases with covalently bound polyether chains at the surface were found to be most suitable for HIC of proteins and t-RNA's.

INTRODUCTION

Reversed phase chromatography with alkyl-silica bonded stationary phases and hydro-organic eluents has been the most widely used technique in HPLC of small molecules (1). It has also

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found employment in the separation of biopolymers on the basis of the differences in their hydrophobic properties and is gaining prominence in analytical work where the high speed and resolving power of the technique is a great advantage (2, 3). In fact, reversed phase chromatography offers a powerful analytical tool complementary to classical gel electrophoresis. However, denaturation of the protein under conditions used in reversed phase chromatography limits the scope of applications when the native protein has to be recovered.

Another approach to biopolymer chromatography on the basis of hydrophobic discrimination has originated from Tiselius' concept of salting-out chromatography. He wrote (4) that "some dyestuffs which are normally not adsorbed on filter paper will do so on addition of salting-out agents, for example ammonium sulfate, and good chromatograms can be obtained by elution with water. Some proteins show this phenomenon too ... and it has been used for the chromatographic separation of some viruses." This prompted his investigation of the effect of salt on the adsorption and the chromatography of proteins (5). More than a score of years later, stationary phases containing mildly hydrophobic functions covalently bound to a suitable inert matrix such as agarose have been introduced for the separation of proteins (6, 7) and subsequently for nucleic acids (8) by a decreasing salt gradient; the technique has been termed hydrophobic interaction chromatography (HIC). At high salt concentrations, biopolymers bind to the stationary phase by hydrophobic interactions which are attenuated upon reducing the salt concentration in the eluent so that they elute in the order of decreasing hydrophobic character. It has been shown that the underlying physico-chemical phenomena are the same in both salting-out and hydrophobic-interaction chromatography of proteins (9) and can be described within the hermeneutics of the solvophobic theory which have been used for the treatment of the thermodynamic aspects of reversed phase chromatography (10).

Although both hydrophobic interaction and reversed phase chromatography have the same intrinsic physico-chemical basis,

they have evolved differently and do not exhibit a great deal of similarities as far as the stationary and mobile phases employed, the operating conditions, and the scope of applications are concerned. Reversed phase chromatography with siliceous stationary phases has been used in the HPLC of small molecules over the last fifteen years and a large body of experimental data of high accuracy are available which facilitate the understanding of the retention process and the design of the separation. On the other hand, hydrophobic interaction chromatography was practiced mainly with stationary phases made of soft polysaccharide gels to separate macromolecular substances by conventional column chromatography and much less retention data of sufficient precision are available to elucidate the physico-chemical aspects of the retention process. The size and complexity of the biopolymer molecules impose additional difficulties in this regard. Therefore, only a few studies attempted to examine the nature of the hydrophobic interactions which are involved in the separation of biopolymers by this technique (11). One of the distinguishing features of HPLC is the use of columns packed with mechanically stable microparticulate stationary phases which afford both high column efficiency and rapid separations. The surface of the rigid porous supports employed in HPLC, however, is usually too "hard" for the chromatography of many biopolymers and their adsorption on such surfaces is usually accompanied by denaturation and sometimes by irreversible adsorption.

Over the last ten years, great advances have been made in chemical modification of surfaces (12, 13) and as a result siliceous and macroporous rigid polymeric stationary phases having "soft" surface are increasingly employed in HPLC of proteins and other biopolymers without untoward effects. In essence, this development has engendered a family of composite stationary phases which combine the mechanical stability of rigid macroporous microparticles and the inert, polar highly hydrated nature of the sacchariferous gels used in traditional column chromatography of biopolymers.

Along these lines high performance columns for size exclusion (14) and for ion exchange chromatography (15, 16) were first developed and have gained increasing popularity in biopolymer chromatography by HPLC and related techniques. Recently stationary phases comprising macroporous siliceous microparticles with a soft or "biocompatible" surface containing mildly hydrophobic binding sites have also been introduced (17-21). As a result, HIC is just about to emerge as a major interactive chromatographic technique for biopolymer separations by HPLC.

In this study certain aspects of hydrophobic interaction chromatography of nucleic acids and proteins are investigated by using siliceous bonded phases having different hydrophobic surface properties. It is noted that hydrophobic interactions are ubiquitous and since all stationary phases employed in biopolymer chromatography contain moieties that exhibit hydrophobic properties under certain circumstances, HIC can be carried out with a wide variety of stationary phases which are otherwise used to separate biopolymers by another retention mechanism (22).

A characteristic feature of HIC is that retention increases with the salt concentration in the eluent. The retention enhancing effect of neutral salts roughly follows the Hofmeister series, which in turn has been shown to represent the effect of salt in increasing the surface tension of aqueous solution (9). As the increase is usually linear with the salt concentration, the salt property of interest here can be conveniently quantified by the molal surface tension increment given by the slope of the plot of surface tension against the salt molality of the solution.

The solvophobic theory predicts that the magnitude of hydrophobic retention is determined by the balance of van der Waals, electrostatic and hydrophobic forces involved in the binding of the eluite to the functional groups at the stationary phase surface. The hydrophobic effect proper is ascribed to the free energy change associated with the contact of the hydrophobic moieties which is given by the product of the hydrophobic contact area and the microthermodynamic surface tension of the medium (23). The subject has been treated elsewhere in detail (9, 24).

For our present purpose it suffices to recall the relationship for the dependence of the logarithmic retention factor on the salt concentration given by:

$$\ln k' = \ln k'_0 - \alpha(m^{1/2})/RT(1+\beta(m^{1/2})) - \Lambda m + \Omega\sigma m \qquad (1)$$

where k'_0 is the retention factor without added salt in the eluent and m is the salt molality. The constants α and β in the Debye term and Λ are described in the text by Edsall and Wyman (25), Ω is the nonpolar contact area between the biopolymer molecule and the stationary phase ligate. σ is the molal surface tension increment of the salt (9).

According to Eqn. 1, the retention factor first decreases with increasing salt concentration because of the effect of salt on electrostatic interactions expressed by the second and third terms which are negative. At further increase in the salt concentration, the retention factor increases and the dependence of log k' on m becomes linear at sufficiently high salt concentrations. Under such conditions, the limiting slope of the log k' vs. m plots is given by $\Omega\sigma - \Lambda$, which we term hydrophobic interaction parameter. Indeed, such plots are frequently linear and according to the above treatment the slope depends on the hydrophobic contact area, the molal surface tension increment of the salt and an electrostatic parameter related to the dipole moment of the biopolymer molecule. A shortcoming of this theory is that specific salt binding by the biomacromolecule (26) is not explicitly treated by Eqn. 1. Nevertheless, it has been found to account for the balance of the hydrophobic and electrostatic interactions underlying protein retention in HIC on stationary phases without fixed charges and when specific effects are absent (22).

EXPERIMENTAL

Materials

Cytochrome c (CYT) from horse heart, α-chymotrypsinogen A (CHY) and ribonuclease A (RNase) both from bovine pancreas,

lysozyme (LYSO) from chicken egg white, ovalbumin, bovine serum albumin, γ-globulin and myoglobin (MYO) from equine skeletal muscle and ferritin (FER) from equine spleen and t-RNA's from E. coli: t-RNA^{val} (VAL), t-RNA^{lys} (LYS), t-RNA^{glu} (GLU) and t-RNA^{phe} (PHE) were supplied by Sigma (St. Louis, MO, USA). 3'-5' oligoriboadenylic acids, $(Ap)_n$ A having n = 1,2,3,4,5, and 7, were from Pharmacia (Piscataway, NJ, USA). Reagent-grade polyethylene glycol (Carbowax), sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, phosphoric acid, and ammonium sulfate, as well as HPLC grade methanol were obtained from Allied-Fisher (Pittsburgh, PA, USA). Polyvinyl alcohol (PVA) of M.W. 3000 was obtained from Polysciences (Warrington, PA, USA). Vydac silica gel support having 300 Å mean pore diameter and 5-μm particle diameter was purchased from the Separation Group (Hesperia, CA, USA). 3-glycidoxypropyltrimethoxysilane was from Aldrich (Milwaukee, WI, USA). Distilled water was prepared with a Barnstead unit.

Instrument

The liquid chromatograph was assembled from a Micromeritics (Norcross, GA, USA) Model 750 pump with a Model 753 ternary solvent mixer and a Model 740 control module. Samples were injected by using a Rheodyne (Berkeley, CA, USA) Model 7010 sampling valve with 20 μl sample loop, and a Model 770R variable-wavelength UV detector (Kratos Analytical, Ramsey, NJ, USA) was used to monitor the column effluent either at 280 nm for proteins or 260 nm for nucleic acids. Chromatograms were recorded with a Model C-R3A integrator (Shimadzu, Columbia, MD, USA).

Columns

Two series of stationary phases have been prepared by using two different supports. For HIC of nucleic acids the support was obtained by reacting Vydac 300 Å silica gel to form at the surface a covalently bound molecular fur of n-propylsilyl groups having

TABLE 1. Stationary Phases Employed in this Study.

Column Code	Ligate		Support Type*
	Chemical structure	MW[D]	
POE 4C	Polyoxyethylene	400	I
POE 1M	Polyoxyethylene	1000	I
POE 1.5M	Polyoxyethylene	1500	I
POE 4M	Polyoxyethylene	4000	I
POE 1M-P	Polyoxyethylene	1000	II
PVA3M	Polyvinylalcohol	3000	II
OAA	Oligoaminoalcohol	290	II

*I = n-propyl silica
II = 1,2-dihydroxy-n-propoxy-n-propyl-silica

reactive functions on the terminal carbon atom of the propyl chain. In a subsequent reaction polyethylene glycol was reacted with the support to obtain a polyether stationary phase in a way similar to that described previously (27). Four products were obtained by using polyethyelene glycols of different molecular weights. For HIC of proteins the support was prepared by reacting the Vydac 300 Å silica gel with 3-glycidoxypropyltrimethoxysilane. Thereafter the polyethylene glycol, polyvinylalcohol and oligoaminoalcohol were reacted with the support (21). The stationary phases prepared in this study are listed in Table 1. Columns were slurry packed into 100 x 4.6 mm No. 316 stainless steel tubes (Handy and Harman, Norristown, PA, USA) using methanol at 8000 psi (28).

Procedures

For non-linear dependence of the log k' values on salt molality, the data were fitted to Eqn. 1 by using SAS-NLIN

software. In the case of linear dependence under the conditions employed, SAS-GLM software was used for data fitting.

RESULTS AND DISCUSSION

Paucity of data characterizing the surface and morphology of the stationary phases as well as the complex structure of biopolymer molecules has impeded the elucidation of the physicochemical phenomena underlying the retention process in HIC beyond the fundamental treatment mentioned above. Undoubtedly peculiarities of the chromatographic system embodying the stationary and mobile phases and the eluites can have a significant effect on the magnitude of hydrophobic interactions and therefore require particular considerations.

In the present study certain aspects of nucleic acid and protein separations by HIC on various stationary phases are examined. The results are expected to facilitate the design of stationary phases. Early works in classical HIC have already concluded that optimum separation of various classes of biopolymer molecules requires stationary phases having various degrees of hydrophobic character (6, 7). It should also be noted that the stratified structure of siliceous bonded phases for HIC gives rise to retention of small molecules by a mechanism different from that involved in biopolymer retention (27).

HIC of Nucleic Acids

The chromatograms illustrated in Fig. 1 show the separation of t-RNA's by using linear gradient elution at decreasing concentration of phosphate buffer, pH 6.3, on POE 4C and POE 4M columns, respectively. It is seen that the elution order of the t-RNA's is the same on both stationary phases as expected since they have the same type of ligates. On the other hand, upon changing the chain length of the weakly hydrophobic polyether ligates, *vide* Table 1, the selectivity of the separation also changes slightly. The POE 4C stationary phase with shorter

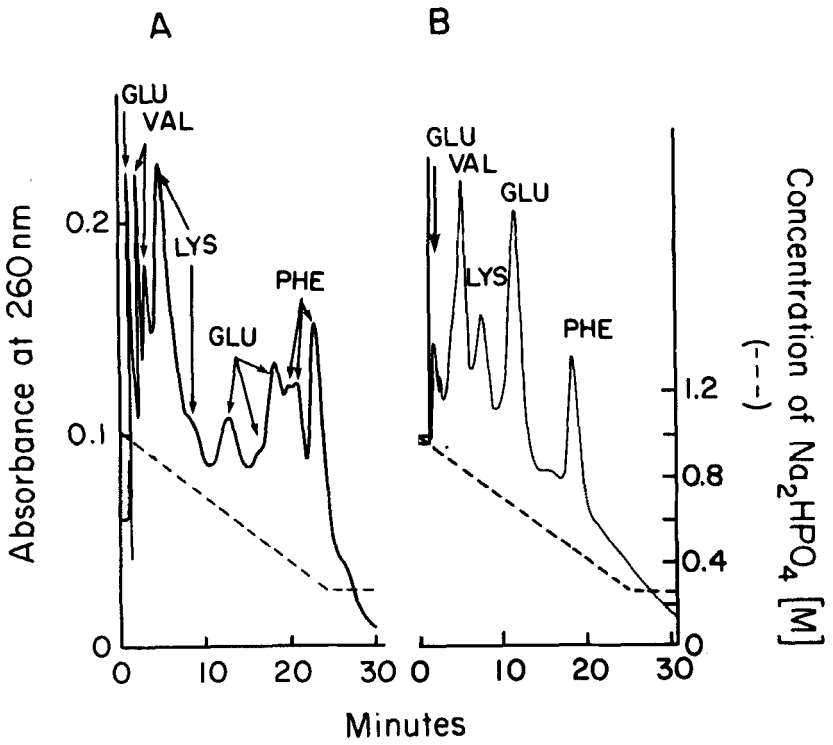

FIGURE 1. Separation of t-RNA's by HIC. Column, 100 x 4.6 mm, (A) POE 4C and (B) POE 4M; flow rate, 1 ml/min; temp., 25°C. Linear gradient in 25 min from 1.0M to 0.25M phosphate buffer, pH 6.3. Column codes and symbols for the eluites are given in the experimental section.

polyether ligates exhibits stronger retentivity and gives higher resolution than the POE 4M stationary phase with ten times longer polyether chains bound to the surface.

The effect of salt concentrations in the eluent on the retention of some t-RNA's was studied isocratically by using phosphate buffer, pH 6.3, at different concentrations as the eluents. Plots of log k' versus the salt concentration yielded straight lines with slopes representing the hydrophobic interaction parameters which are listed in Table 2. As seen in

Table 2 the slope for t-RNA^{val} increases whereas the slope for t-RNA^{lys} decreases upon increasing the length of the bound polyether chains. This suggests, in view of Eqn. 1, that the hydrophobic contact area of t-RNA^{lys} is smaller with POE 4M than with POE 4C or that some electrostatic effect accounts for the smaller slope.

The retention of small oligoriboadenylic acid homologues, $(Ap)_nA$, having n ranging from 1 to 7 was also studied on POE 4C and POE 1M stationary phases at different concentrations of the phosphate buffer, pH 6.3, in the eluent. In agreement with the solvophobic theory, plots of log k' versus the number of adenylyl phosphate residues, n, were linear at different concentrations of the eluting salt. The slope of the lines thus obtained represent the retention increments of an adenylyl phosphate unit, τ_{Ap}, which by definition (27, 29) equals the logarithm of the relative retention of a given oligoadenylic acid $(A_p)_nA$ with respect to the antecedent member of the homologous series $(A_p)_{n-1}A$. As shown in Fig. 2, the τ_{Ap} values depend on the salt concentration in a linear fashion as expected from the solvophobic theory. The retention increments of adenylyl phosphate residues are essentially the same on both stationary phases, i.e., they are independent of the length of the polyether chains over a wide range of salt concentration. Plots of log k' measured on both POE 4C and POE 1M columns against the salt concentration also yielded straight lines, and as shown in Fig. 3, the slopes representing the hydrophobic interactions parameter were linearly dependent on the number of adenylyl phosphate residues in the oligonucleotides under investigation. According to the theory, the slopes of the lines in Fig. 3 are proportional to the hydrophobic contact area between a residue and the stationary phase ligates. The respective slopes for POE 4C and POE 1M are 0.18 and 0.16 indicating that the incremental contact area for a residue is practically independent of the length of the polyether ligates on the two stationary phases. It is possible, however, that the relatively small oligonucleotides are retained not by the polyether ligates but by the hydrocarbonaceous sublayer of this

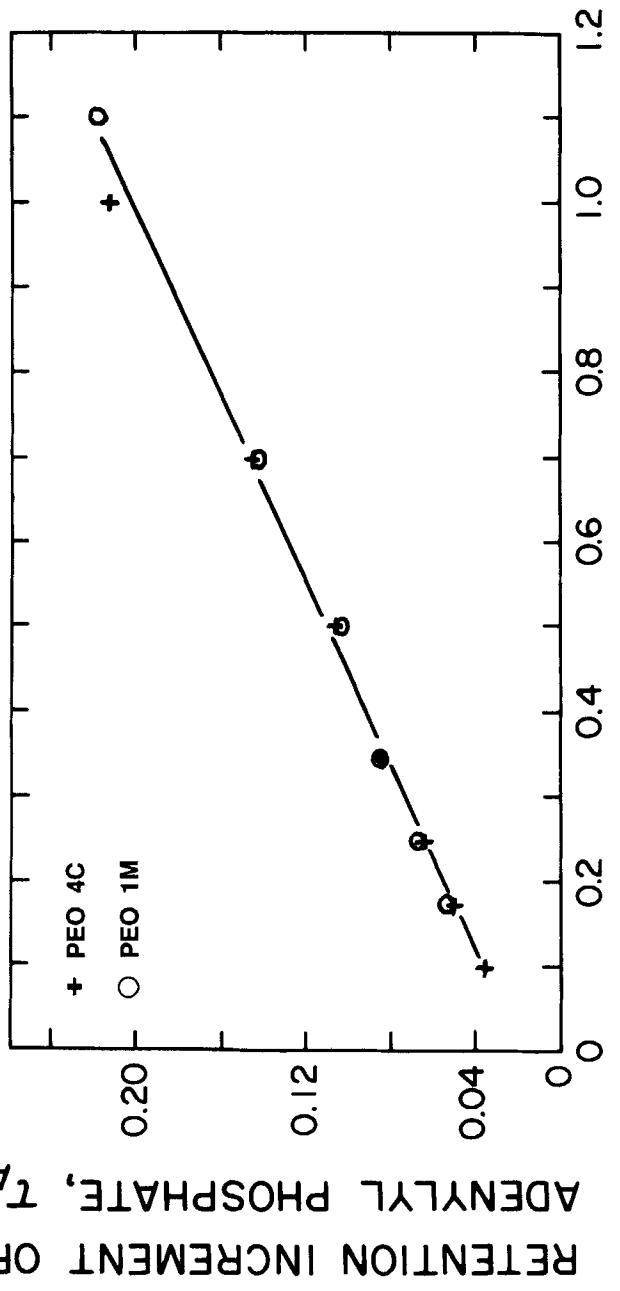

FIGURE 2. Plot of retention increment of adenylyl phosphate residues against the concentration of phosphate buffer, pH 6.3, used as the eluent with columns POE 4C and POE 1M. Column codes are given in the experimental section.

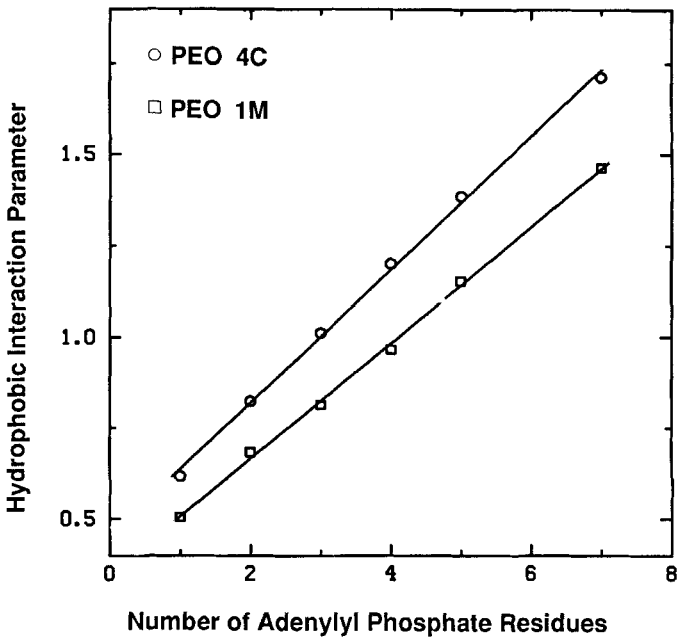

FIGURE 3. Plot of the hydrophobic interaction parameter against the number of adenylyl phosphate residues. The data were obtained on columns POE 4C and POE 1M with phosphate buffer, pH 6.3, having different concentrations and the solid lines were obtained by linear regression. Column codes are given in the experimental section.

type of stratified stationary phase as discussed in an earlier report (27). If this is the case, no significant differences in the retention increments are expected.

HIC of proteins

The above discussed stationary phases that were prepared primarily for the separation of t-RNA's were also employed in HIC of proteins. As shown in Table 1 these stationary phases contain polyether moieties attached directly to n-propyl-silica. These stationary phases have a rather strong hydrophobic character and by using 0.1M phosphate buffer, pH 6.3 no protein was eluted from

POE 4C and only ribonuclease A and cytochrome c were eluted from POE 1M. The stationary phase POE 4M which contains the longest polyether chains (MW 4000) was used for the chromatography of small proteins with decreasing salt gradient but at a much lower starting salt concentration in the eluent than that used with a relatively weaker hydrophobic phase designed for HIC of larger proteins (21). Separation of proteins on the POE 4M column by using a decreasing phosphate gradient with or without polyethylene glycol in the eluent is illustrated by the chromatograms in Fig. 4. By addition of Carbowax 4000 to the gradient former, larger and more hydrophobic proteins, such as BSA, were also eluted with decreasing salt gradient. This observation and the examination of the chromatograms in Fig. 4 show that the hydrophobic retentivity of the stationary phase for proteins can be reduced by the addition of polyethylene glycol to the eluent. This effect is shown also in Fig. 5. Thus, polyethylene glycol at low concentrations in the eluent is a useful adjunct to separate proteins of widely different hydrophobic character on a given stationary phase by HIC.

The stationary phases were compared for their retention of proteins in isocratic elution at different phosphate concentrations in the mobile phase at pH 6.3 and yielded straight line plots of log k' against the salt concentration. The slopes of the lines representing the pertinent hydrophobic interaction parameter are listed in Table 2. For ovalbumin and lysozyme these parameters are found to increase with the chain length of the stationary phase ligates indicating an increase in the hydrophobic contact area upon increasing the length of the bound polyether chains. On the other hand, the hydrophobic interaction parameter for ribonuclease is practically independent of the length of the polyether ligate.

These stationary phases were also employed for the separation of oligo-phenylalanines containing up to 4 residues by isocratic elution with 0.5 M phosphate buffer, pH 6.3. As with the oligoriboadenylic acids, the retention increments of the

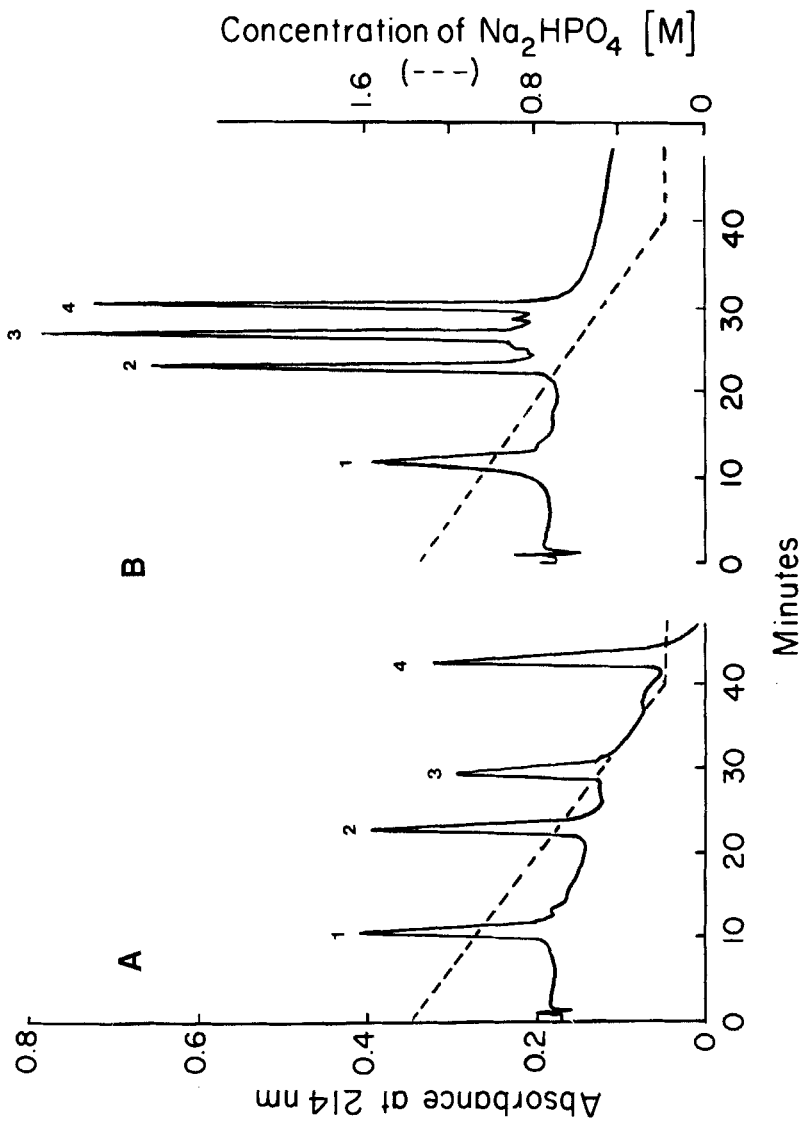

FIGURE 4. Separation of proteins by HIC. Column, 100 x 4.6 mm, POE 4M; flow rate, 1 ml/min; temp., 25°C. Linear gradient in 40 min from 1.2 to 0.25M phosphate buffer, pH 6.3. (A) without and (B) with 0.2% (w/w) Carbowax 4000 in the eluent. Eluites: ribonuclease A (1), lysozyme (2), trypsinogen (3), and α-chymotrypsinogen A (4). Column code is given in the experimental section.

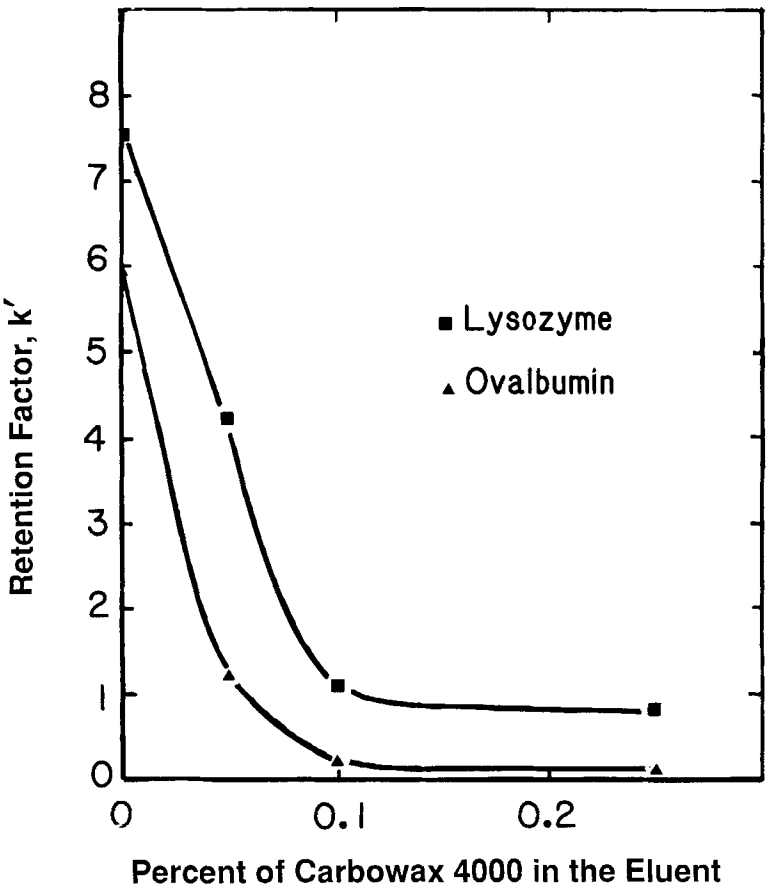

FIGURE 5. Plot of the retention factor k' of proteins against the concentration of Carbowax 4000 in the eluent. Column, 100 x 4.6 mm I.D., POE 4M. Isocratic elution with 1.0M phosphate buffer, pH 6.3.

phenylalanyl residues are practically independent of the length of the polyether ligates and they are found to be 0.82 and 0.89 on POE 1.5M and POE 4M columns, respectively. It is likely that small molecules such as the short oligopeptides have almost equal access to the hydrocarbonaceous sublayer at the surface of the stationary phases, and thus their retention behavior is not

TABLE 2. Hydrophobic Interaction Parameters as Measured by the Limiting Slopes of log k' Against Na_2HPO_4 Molality at High Salt Concentration in the Eluent, pH 6.3, for t-RNA's and Proteins on Different Stationary Phases.

	Stationary Phase			
Eluite	POE 4C	POE 1M	POE 1.5M	POE 4M
t-RNA^{val}	1.00	1.38	NM	NM
t-RNA^{lys}	1.99	1.72	NM	NM
Ribonuclease A	NE	2.12	2.52	2.54
Lysozyme	NE	NE	1.66	3.20
Ovalbumin	NE	NE	4.77	6.67

NM = not measured.
NE = not eluted.

affected significantly by the size of the hydrophilic polyether moieties. On the other hand, the proteins are believed to interact only with the bound polyether moiety which effectively shrouds the surface region of the stationary phase for bulky protein molecules.

Further studies were carried out with another set of stationary phases, vide Table 1, which contained weakly hydrophobic functions bound to dihydroxypropoxypropyl-silica phase. These stationary phases exhibited relatively weak hydrophobic character in comparison to the column materials discussed above. Chromatograms of proteins obtained with such stationary phases containing either polyvinyl alcohol or polyoxyethylene moieties are shown in Fig. 6. In both cases, the elution order was the same but selectivities were different and sharper peaks were obtained on the polyether stationary phase. This finding supports the observations (20, 21, 30) that stationary phases with polyether ligates result in efficient

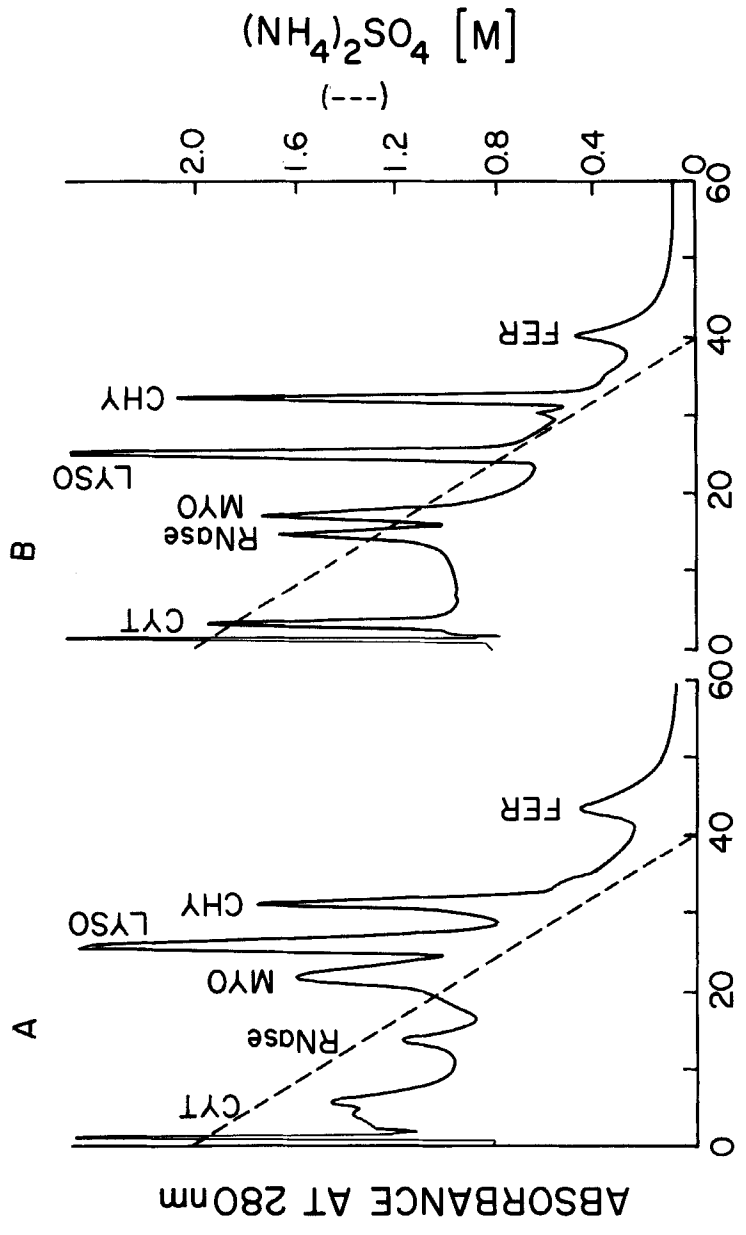

FIGURE 6. Separation of proteins by HIC. Column, 100 x 4.6 mm, (A) PVA 3M and (B) PEG 1M-P; flow rate, 1 ml/min; temp., 25°C. Linear gradient over 40 min with ammonium sulfate in 0.1M phosphate buffer, pH 7.0. Column codes and symbols for the eluites are given in the experimental section.

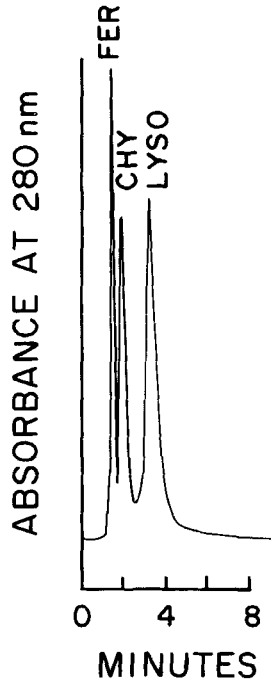

FIGURE 7. Separation of proteins by isocratic elution. Column, 100 x 4.6 mm, OAA; flow rate 1 ml/min; temp., 25°C. Mobile phase, 0.1 M phosphate buffer, pH 7.0. Column codes and symbols for the eluites are given in the experimental section.

columns for separations by HIC. Isocratic elution with 0.1M phosphate buffer was also used for the separation of some proteins on such weakly hydrophobic stationary phases as seen in Fig. 7 and the order of elution is reversed with respect to that obtained in decreasing $(NH_4)_2SO_4$ gradient in the same buffer at pH 7.0. Ferritin is excluded at the intermediate salt concentration used in the eluent whereas lysozyme and α-chymotrypsinogen A are retained by hydrophobic interactions.

The effect of salt concentration on protein retention on these stationary phases was examined under isocratic elution conditions by using 0.1M phosphate buffer, pH 7.0, containing different concentrations of ammonium sulfate. The results are

depicted in Fig. 8 which shows plots of log k' obtained with various proteins on three stationary phases against the molality of $(NH_4)_2SO_4$ in the eluent. In all cases a good fit of the data points is obtained to Eqn. 1. On PVA 3M stationary phase the retention of proteins having high pI values such as lysozyme, α-chymotrypsinogen A, cytochrome c and ribonuclease A first decreased then increased with increasing salt concentration and at sufficiently high salt concentrations the plots yielded a straight line as suggested by Eqn 1. However on OAA and POE 1M-P stationary phases only lysozyme exhibited this behavior and for all other proteins log k' is linearly dependent on the salt concentration under the conditions of the experiment. The hydrophobic interaction parameters given by the slopes of these lines or the limiting slopes obtained with the nonlinear systems at high salt concentration are listed in Table 3. Comparison of the results presented in Fig. 8 and in Table 3 shows that non-linear salt dependence of the logarithmic retention is found under the conditions of the present experiments when the strength of hydrophobic interactions as measured by the magnitude of the limiting slopes is relatively small.

As seen in Table 3, the slopes correlate with the molecular weight of the proteins and for all stationary phases investigated here, the hydrophobic interaction parameter increases with the size of the proteinaceous eluite. This observation is in good agreement with the results obtained with the other set of stationary phases listed in Table 2. In view of Eqn. 1, they suggest that the hydrophobic contact area upon binding to the stationary phase ligates increases with the molecular weight of the protein. This is not unexpected and inndeed, the retention order of proteins in HIC is roughly that of increasing molecular weight. This is similar to the retention behavior of homologous eluites in reversed phase chromatography.

Examination of the data in Table 3 also shows that the hydrophobic interaction parameters are generally the greatest and smallest for POE 1M-P and PVA 3M stationary phases, respectively

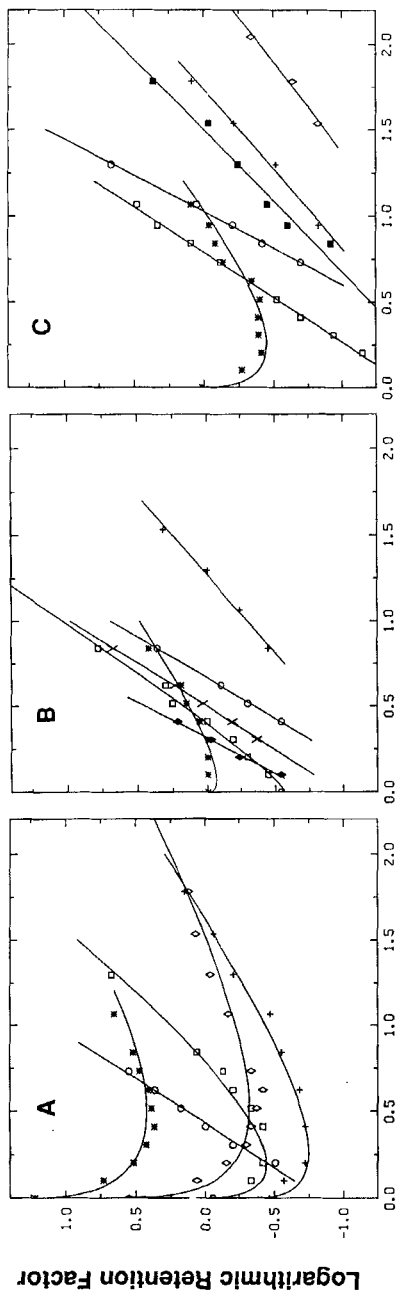

FIGURE 8. Plots of the logarithmic retention factors of various proteins measured on different columns against the molality of ammonium sulfate in the eluent. Columns: A, PVA 3M; B, OAA; C, POE 1M. Mobile phase, 0.1M phosphate buffer, pH 7.0. Eluites: (+), ribonuclease; (◇) cytochrome c; (■), myoglobin; (□), α-chymotrypsinogen; (O), ovalbumin; (◆) γ-globulin; (X), bovine serum albumin; (*), lysozyme. Column codes are given in the experimental section.

TABLE 3. Hydrophobic Interaction Parameters as Measured by the Limiting Slopes of the Plots of log k' vs. Ammonium Sulfate Molality at High Salt Concentration in the Eluent, pH 7.0, for Various Proteins and Stationary Phases. The Molecular Weights and the Isoelectric Points of the Proteins are also Listed.

PROTEIN			STATIONARY PHASE		
NAME	MW	pI	PVA 3M	OAA	POE 1M-P
Cytochrome C	12,200	10.6	0.77	NR	0.96
Ribonuclease A	13,700	9.4	1.02	1.09	1.09
Lysozyme	14,000	11.0	1.22	0.59	1.29
Myoglobin	17,500	7.1	NM	NM	1.22
α-Chymotrypsinogen A	25,500	9.5	1.86	1.90	1.91
Ovalbumin	44,000	4.7	1.93	2.08	2.38
Bovine serum albumin	65,000	4.4	NM	1.96	NM
γ-Globulin	160,000	---	NM	2.41	NM

NR = not retained.
NM = not measured.

and are intermediate for OAA. The low value of the hydrophobic interaction parameter obtained from retention data for the positively charged lysozyme on the OAA stationary phase which has ligates containing amino functions is likely to be due to electrostatic repulsion which persists even at the high salt concentrations used in the eluent. This is supported by the observation that cytochrome c, another strongly basic protein, was not retained on the OAA stationary phase using salt concentrations as high as 2.2M ammonium sulfate in the eluent.

The effect of salt on the retention in hydrophobic interaction chromatography resembles the salting-out of proteins and it may be tempting to consider the mechanism underlying the retention as salting-out. Although there is a certain similarity

between the salting-out constant (9) and the hydrophobic interaction parameter as defined above, they are different physico-chemical parameters. The salting-out constant is an intrinsic property of the protein and the salt only. On the other hand, the results presented in Tables 2 and 3 offer ample evidence that the magnitude of the hydrophobic interaction parameter for a given protein is greatly influence by the chemical nature and molecular size of the stationary phase ligates. This is in agreement with our view that retention in HIC is due to the salt mediated interaction between the biopolymer molecule and the mildly hydrophobic binding sites of the stationary phase in contradistinction to the salting-out process that is the result of protein-protein interactions. The term "salting-out chromatography" suggested by Tiselius' work (4, 5), therefore, could be acceptable only in the broadest sense of the word. Since the stationary phase plays an essential role in determining the magnitude of retention and selectivity in HIC, efficient separation of various types of biopolymers by this technique is likely to require a family of columns having different shades of hydrophobic properties.

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