Journal of Chromatography, 499 (1990) 141–152
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1652

BINARY AND TERNARY SALT GRADIENTS IN HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF PROTEINS

ZIAD EL RASSI*, LUCILA F. DE OCAMPO and MARIA D. BACOLOD
Department of Chemistry, Oklahoma State University, Stillwater, OK 74078-0447 (U.S.A.)

SUMMARY

Hydrophobic-interaction chromatography of mixtures of acidic and basic proteins having a wide range of molecular weights and hydrophobic character was carried out by using binary and ternary salt gradients. Chaotropic and antichaotropic salts as well as organic salts were incorporated in the eluents. The stationary phase consisted of macroporous silica with surface-bound polyether moieties. At constant eluent surface tension, gradient elution with two or three aqueous salt solutions was found to be superior to single-salt gradients in modulating hydrophobic-interaction chromatography retention and selectivity. The effect was attributed to the competitive salt-specific binding to the protein molecule and/or the stationary phase surface. Chaotropic/antichaotropic salt gradient systems exhibited vastly different selectivities upon changing the nature and concentrations of salts in the eluents. In general, the retention of basic proteins increased while that of acidic proteins either decreased or remained unchanged with the use of chaotropic salts. At the same surface tension of the eluent, KSCN and $KClO_4$ yielded different selectivities. The addition of organic salts, such as tetrabutylammonium bromide was found to be suitable for the separation of proteins having a wide range of isoelectric points.

INTRODUCTION

Hydrophobic-interaction chromatography (HIC) with rigid, microparticulate stationary phases is increasingly used for the analysis and purification of proteins. Due to the weakly hydrophobic character of the stationary phase, proteins are first adsorbed to the column, equilibrated with an eluent of relatively high salt concentration at or near neutral pH, and subsequently eluted by a decreasing salt concentration gradient.

The retention behavior of proteins in HIC can be described by the thermodynamic model of Horváth and co-workers[1,2] based on the solvophobic theory of Sinanoğlu and co-workers[3,4]. According to the model, at relatively high salt concentrations in the eluent the retention increases with the salt molality and at constant salt concentration with the molal surface tension increment of the salt used in the aqueous eluent. The validity of the model requires that there be no specific binding of the salt

0021-9673/90/$03.50

to the protein molecule. The retention–surface tension dependency parallels the Hofmeister series, which is based on the ability of the salts to cause precipitation of proteins from aqueous solutions. Proteins are more retained on an HIC column with antichaotropic salts (*e.g.*, phosphates, sulfates, tartrates and citrates) than with chaotropic salts (*e.g.*, perchlorates and thiocyanates). The solvophobic theory was successfully applied to treat the retention behavior of proteins in HIC as a function of the salt concentration in the eluent[2,5], the hydrophobic character of the proteins[6], and the hydrophobicity of the stationary phase ligates[7].

Selectivity in HIC is altered by means of several operating parameters, including the nature and concentration of the salt used in the eluent[8,9], slope of the salt gradient[10,11], eluent pH[12–14], addition of organic solvents[15,16], denaturing agents[12] and surfactants[17] to the eluent, column temperature[15,18] and the nature of the stationary phase ligates[19–21].

This study is concerned with investigating the potential of binary and ternary salt gradients in modulating retention and selectivity in HIC of proteins. The terms binary and ternary salt gradients refer to changing the composition of two and three salts in the eluent, respectively. The significance of such an approach resides in its analytical and preparative applicability to a wide range of separation problems of relevance to many areas of the life sciences.

EXPERIMENTAL

Instrumentation

The liquid chromatograph was assembled from an LDC/Milton Roy (Riviera Beach, FL, U.S.A.), Model CM4000, solvent delivery pump with a dual-beam variable-wavelength detector, Model SpectroMonitor 3100. The column effluent was monitored at 280 nm. Samples were injected by a Model 7125 sampling valve with a 100-μl sample loop (Rheodyne, Cotati, CA, U.S.A.). Chromatograms were recorded with a Shimadzu (Columbia, MD, U.S.A.) Model C-R6A integrator, interfaced with a single floppy disk drive.

Columns

Zorbax PSM 300, a spherical silica, having mean particle and pore diameters of 7.5 μm and 300 Å, respectively, was obtained from DuPont (Wilmington, DE, U.S.A.). The polyether stationary phase was prepared by covalent attachment of polyethyelene glycol (PEG) of average mol. wt. 1000 to the surface of the Zorbax silica gel, using well-established procedures[6,22]. The silica-bound polyether was packed from a methanol slurry at 8000 p.s.i., using a Shandon column packer instrument (Keystone Scientific, Bellefonte, PA, U.S.A.). All columns were made of 100 × 4.6 mm I.D. No. 316 stainless-steel tubes (Alltech, Deerfield, IL, U.S.A.).

Materials

The proteins used in this study are listed in Table I. They were purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade hydrochloric acid, glacial acetic acid, phosphoric acid, sodium hydroxide, ammonium sulfate, sodium chloride, disodium hydrogen phosphate, sodium tartrate, sodium citrate, potassium thiocyanate, potassium perchlorate and methanol (HPLC grade) were obtained from Fisher (Pitts-

burgh, PA, U.S.A.) or J. T. Baker (Phillipsburg, NJ, U.S.A.). Tetrabutylammonium bromide was obtained from Aldrich (Milwaukee, WI, U.S.A.).

Procedure

Proteins were dissolved in water at concentration of 5 mg/ml for fetuin, ovalbumin, β-lactoglobulin A, and ribonuclease A and at 2 mg/ml for lysozyme, cytochrome *c*, and α-chymotrypsinogen A. They were chromatographed by using 30-min linear gradient elution of one, two, or three salts at room temperature and a flow-rate of 1.0 ml/min.

The surface tension, γ, of aqueous salt solutions was estimated from the molal salt concentration, m, the molal surface tension increment, σ, of the salt, and the surface tension of pure water, γ_0 (72 dynes/cm at 25°C), using the equation $\gamma = \gamma_0 + \sigma m$. An extensive listing of molal surface tension increments of salts can be found in ref. 1.

RESULTS AND DISCUSSION

A mixture of seven proteins (Table I), having a wide range of isoelectric points, molecular weights and hydrophobic character was chromatographed on a silica-bound PEG (mol.wt = 1000) column by using single, binary, or ternary salt gradients. In this regard, several salts were used including ammonium sulfate, sodium tartrate, sodium citrate, sodium chloride, potassium perchlorate, potassium thiocyanate and tetrabutylammonium bromide. In all cases, a 30-min linear gradient was used and the background buffer was 50 m*M* phosphate (pH 6.5).

Linear gradients of decreasing ammonium sulfate, sodium tartrate or sodium citrate concentrations in the eluent (single-salt gradients) were first used in order to evaluate their effectiveness in HIC of the proteins under investigation and to establish a reference to which the retention data obtained by using binary and ternary salt gradients could be compared. Binary salt gradients were carried out (i) by decreasing the concentration of two antichaotropic salts in the eluent, (ii) by decreasing the concentration of an antichaotropic salt while increasing the concentration of a chaotropic salt in the eluent or (iii) by decreasing the concentration of an antichaotropic salt while increasing the concentration of an organic salt in the eluent. Ternary salt gradients were run by decreasing the concentration of two antichaotropic salts while increasing the concentration of a third salt, a chaotropic salt.

TABLE I

PROTEINS USED IN THIS STUDY

Protein	*Symbol*	*Mol. wt.*	*pI*	*Source*
Cytochrome *c*	a	12 200	10.6	Horse heart
Ribonuclease A	b	13 700	9.5	Bovine pancreas
β-Lactoglobulin A	c	35 000	5.1	Bovine milk
Lysozyme	d	14 000	11.0	Chicken egg white
Ovalbumin	e	44 000	4.7	Chicken egg
α-Chymotrypsinogen A	f	25 500	9.5	Bovine pancreas
Fetuin	g	48 400	3.2–4.4	Fetal calf serum

The adjusted retention volumes of proteins, measured with single-salt gradients at decreasing ammonium sulfate, sodium tartrate or sodium citrate concentrations in the eluent, are reported in Table II (gradients I, III and V). Sodium citrate and tartrate yielded higher retention than ammonium sulfate at approximately the same eluent surface tension (76.9 dynes/cm). The effectiveness of these salts in protein adsorption on the HIC matrix increased in the order: ammonium sulfate < sodium tartrate < sodium citrate. The adjusted retention volumes of basic proteins, such as cytochrome *c*, ribonuclease A and lysozyme increased to a larger extent than those of other proteins in the sample when changing ammonium sulfate to either sodium citrate or sodium tartrate. A reversal in the elution order for the eluite pair lysozyme and ovalbumin was observed with both sodium citrate and tartrate, when compared with ammonium sulfate. It is believed that both citrate and tartrate (but to a larger extent citrate) form ion pairs with the proteins and especially the basic ones, which make these proteins more hydrophobic than with ammonium sulfate. At the pH of the experiment, *i.e.* pH 6.5, citrates are trivalent anions (pK_{a1} = 3.06, pK_{a2} = 4.74 and pK_{a3} = 5.40), whereas tartrates are divalent anions (pK_{a1} = 2.96 and pK_{a2} = 4.24).

As expected, binary salt gradients of decreasing concentrations of both ammonium sulfate and sodium citrate in the eluent, from 1.0 to 0 *M* and from 0.69 to 0 *M*, respectively, yielded an elution pattern significantly different from that obtained by using either ammonium sulfate or sodium citrate gradients while maintaining the surface tension of the eluents constant (compare gradient IX with gradients I and V in Table II). The adjusted retention volumes of fetuin and α-chymotrypsinogen A in-

TABLE II

ADJUSTED RETENTION VOLUMES OF PROTEINS

Polyether column (100 × 4.6 mm I.D.) with linear gradient elution in 30 min. The background buffer was 50 m*M* phosphate (pH 6.5); flow-rate, 1 ml/min; temperature, 25°C. Gradients: I = ammonium sulfate gradient from 2.0 to 0 *M*; II = ammonium sulfate and potassium thiocyanate gradients from 2.0 to 0 *M* and from 0 to 0.5 *M*, respectively; III = sodium tartrate gradient from 1.70 to 0 *M*; IV = sodium tartrate and potassium thiocyanate gradients from 1.70 to 0 *M* and from 0 to 0.5 *M*, respectively; V = sodium citrate gradient from 1.38 to 0 *M*; VI = sodium citrate and potassium thiocyanate gradients from 1.38 to 0 *M* and from 0 to 0.5 *M*, respectively; VII = ammonium sulfate and sodium tartrate gradients from 1.0 to 0 *M* and 0.85 *M* to 0 *M*, respectively; VIII = ammonium sulfate, sodium tartrate and potassium thiocyanate gradients from 1.0 to 0 *M*, from 0.85 to 0 *M* and from 0 *M* to 0.5 *M*, respectively; IX = ammonium sulfate and sodium citrate gradients from 1.0 *M* to 0 *M* and from 0.69 *M* to 0 *M*, respectively; X = ammonium sulfate, sodium citrate and potassium thiocyanate gradients from 1.0 to 0 *M*, from 0.69 to 0 *M* and from 0 to 0.5 *M*, respectively.

Protein	*Gradient*									
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*
Cytochrome *c*	0.30	0.85	0.70	1.40	2.13	5.71	0.40	0.77	0.53	1.93
Ribonuclease A	5.77	5.59	7.50	9.50	12.62	12.64	6.93	7.85	9.02	9.98
β-Lactoglobulin A	10.98	13.27	13.80	13.70	15.07	15.48	11.36	13.22	13.98	14.55
Lysozyme	12.66	22.10	17.13	24.27	19.05	26.95	17.13	23.96	18.60	24.96
Ovalbumin	13.28	14.72	14.18	13.70	16.97	16.69	14.39	14.63	17.00	15.98
α-Chymotrypsinogen A	20.14	24.63	21.60	25.40	23.26	26.20	21.10	23.70	23.69	25.37
Fetuin	19.97	22.89	22.12	22.60	23.25	24.97	21.60	22.65	24.43	24.02

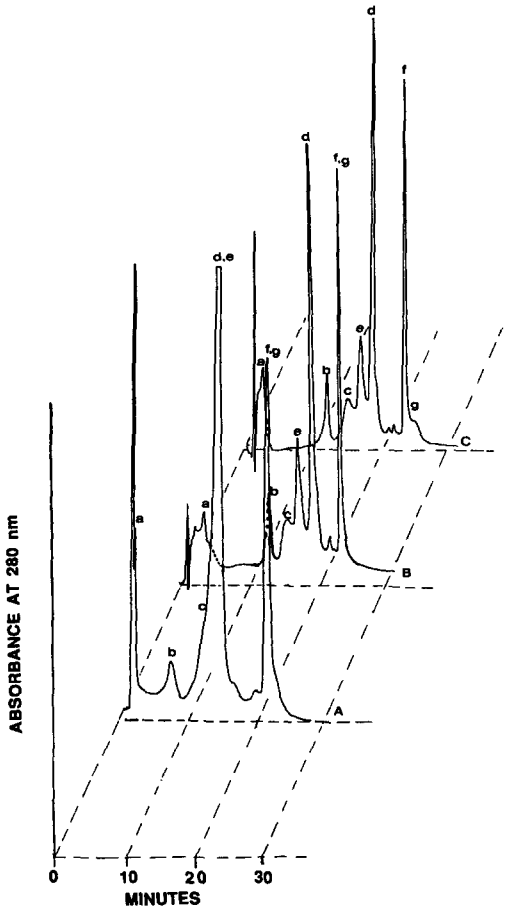

Fig. 1. Hydrophobic-interaction chromatography of proteins. Column, silica-bound polyether, 100 × 4.6 mm I.D.; temperature 25°C; flow-rate, 1 ml/min. (A) Ammonium sulfate gradient from 2.0 *M* to 0 *M*; (B) sodium citrate gradient from 1.38 to 0 *M*; (C) binary salt gradient of ammonium sulfate and sodium citrate from 0.375 to 0 *M* and from 1.125 to 0 *M*, respectively. In all cases, a linear gradient of 30 min was used. The background buffer was 50 m*M* phosphate (pH 6.5). For symbols see Table I.

creased and a reversal in their elution order was observed with the binary salt gradient as compared to ammonium sulfate or sodium citrate gradients. Whereas the adjusted retention volume of ovalbumin, measured with the binary salt gradient, equaled that obtained by using sodium citrate gradient, the retention volumes of other components of the protein mixture ranked between those obtained by using individual salt gradients of ammonium sulfate and sodium citrate, respectively.

Upon changing the composition of ammonium sulfate and sodium citrate in the starting eluent to 0.375 *M* and 1.125 *M*, respectively, while keeping the eluent surface tension roughly the same as in the preceding experiment, the elution profile of proteins under investigation became slightly different. As seen in Fig. 1C, with the binary salt gradient, the seven proteins were retained to an extent closer to that obtained by

using a sodium citrate gradient than an ammonium sulfate gradient. However, the eluite pairs ribonuclease A and β-lactoglobulin A, ovalbumin and β-lactoglobulin A, and fetuin and α-chymotrypsinogen A are better resolved with the binary salt gradient than with single-salt gradients at virtually equal eluent surface tension (compare Fig. 1C with Figs. 1A and 1B).

Also, binary salt gradients of decreasing concentrations of both ammonium sulfate and sodium tartrate in the eluent (gradient VII in Table II) produced an elution pattern different from that obtained by using single-salt gradients of ammonium sulfate (gradient I) or sodium tartrate (gradient III) at practically the same eluent surface tension of *ca.* 76.9 dynes/cm. Whereas an increase in the adjusted retention volume of ovalbumin was observed with the binary salt gradient as compared to ammonium sulfate or sodium tartrate gradients, the retention of lysozyme measured with the binary salt gradient was identical to that obtained by using sodium tartrate

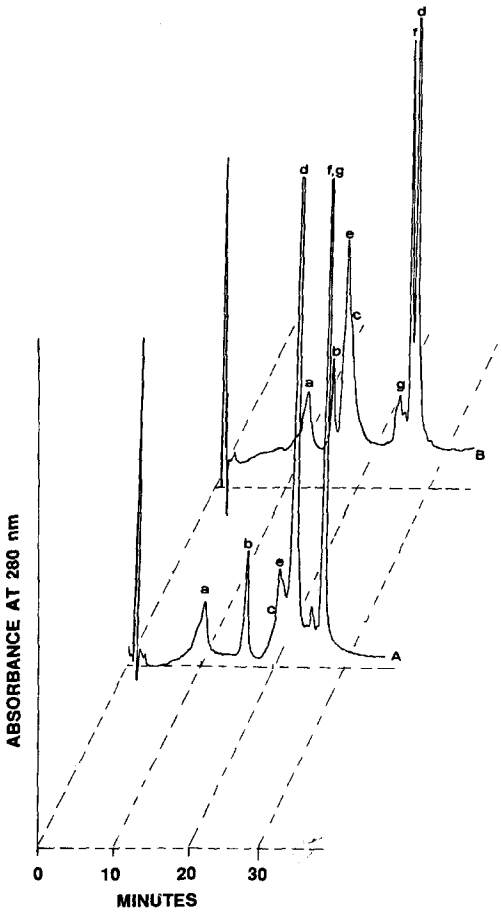

Fig. 2. Hydrophobic-interaction chromatography of proteins. (A) Sodium citrate gradient from 1.5 to 0 *M*; (B) binary salt gradient of sodium citrate and potassium thiocyanate from 1.5 to 0 *M* and from 0 to 0.5 *M*, respectively. Other conditions as in Fig. 1.

gradient. The adjusted retention volumes of other proteins, measured with the binary salt gradient, fell in between those obtained by using single-salt gradients. However, the elution order with the binary salt gradient paralleled that obtained by using a sodium tartrate gradient.

Salt selectivity in HIC arises from quantitative differences in salt-specific binding to proteins[5,23–25] and/or from differential hydration of both stationary phase and protein surfaces. In the light of the above results, by using binary salt gradients of different salt composition and types of salts, different selectivities can be generated. This may be due to competitive, salt-specific binding to protein and/or the stationary phase.

Other types of binary salt gradients were also tested for their effectiveness in modulating retentivity and selectivity in HIC. Fig. 2B depicts the chromatogram obtained by using a binary salt gradient of decreasing sodium citrate and increasing potassium thiocyanate concentrations in the eluent. Comparison of this chromatogram to that in Fig. 2A, which was obtained by using sodium citrate gradient, reveals that the binary salt gradient has resolved most of the proteins in the mixture and yielded a different selectivity, as manifested by the reversal in the elution order of the eluite pair β-lactoglobulin A and ovalbumin and the pair lysozyme and α-chymotrypsinogen A as well as the pair fetuin and α-chymotrypsinogen A. The addition of 0.5 *M* KSCN to the gradient former, *i.e.* the strong eluent, increased the surface tension by only 0.22 dynes/cm. However, lysozyme, and to lesser extent cytochrome *c* and α-chymotrypsinogen A were much more retained in the presence than in the absence of KSCN. In addition, whereas the retention of ribonuclease A was not affected by the presence of KSCN, acidic proteins, such as ovalbumin and β-lactoglobulin A were less retained with than without KSCN in the gradient former.

Keeping the concentration of KSCN in the gradient former identical to that used in the above binary salt gradient, but using 1.38 *M* instead of 1.5 *M* sodium citrate in the starting eluent (gradient VI in Table II) produced an elution pattern different from that in Fig. 2B as far as the elution order of the eluite pair ovalbumin and β-lactoglobulin A is concerned. The selectivity of the sodium citrate–KSCN gradient system toward the proteins has also changed upon reducing the concentration of KSCN from 0.5 *M* to 0.2 *M* in the gradient former. Binary salt gradients of decreasing antichaotropic salt concentration and increasing chaotropic salt concentration in the eluent offer the opportunity for the optimization of selectivity in HIC, because changing the concentration of the salts in the starting eluent and the gradient former leads to widely different selectivities.

When using chaotropic/antichaotropic salt gradient systems, sample selectivity can also be varied by keeping the eluent surface tension constant while exchanging one antichaotropic salt for another in the starting eluent. The adjusted retention volumes of proteins, measured with ammonium sulfate–, sodium tartrate– or sodium citrate–KSCN gradients, are summarized in Table II (gradients II, IV and VI, respectively). The adjusted retention volumes of proteins were the highest with the sodium citrate–KSCN gradient and the lowest with the ammonium sulfate–KSCN gradient. When using a citrate–KSCN gradient the elution order of the following eluites was: fetuin < α-chymotrypsinogen A < lysozyme. It changed to fetuin < lysozyme < α-chymotrypsinogen A and to lysozyme < fetuin < α-chymotrypsinogen A when using tartrate–KSCN and sulfate–KSCN gradients, respectively, instead of the sodium citrate–KSCN gradient.

In order to examine the influence of the nature of the chaotropic salt on HIC selectivity in a binary salt gradient system, gradient elution of decreasing ammonium sulfate concentration and increasing $KClO_4$ concentration was carried out. The concentration of $KClO_4$ in the gradient former was adjusted to give approximately the same surface tension as with KSCN (*ca.* 72.22 dynes/cm). The chromatogram ob-

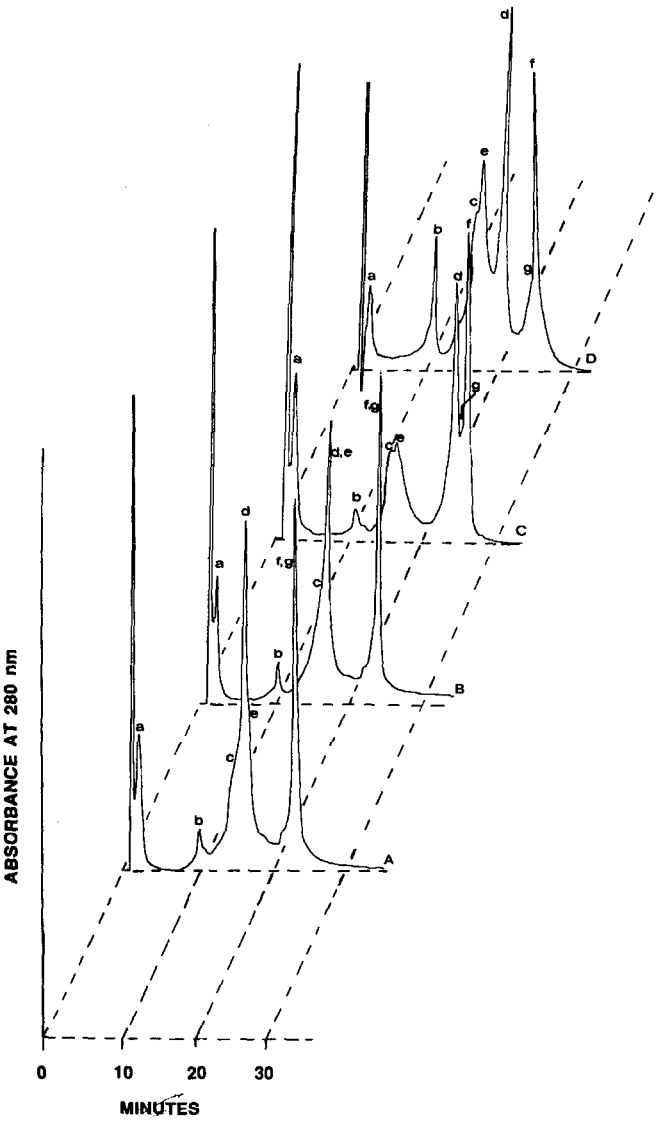

Fig. 3. Hydrophobic-interaction chromatography of proteins. (A) Ammonium sulfate gradient from 2.16 to 0 *M*; (B) binary salt gradient of ammonium sulfate and sodium chloride from 2.16 to 0 *M* and from 0 to 0.15 *M*, respectively; (C) binary salt gradient of ammonium sulfate and potassium thiocyanate from 2.16 to 0 *M* and from 0 to 0.5 *M*, respectively; (D) binary salt gradient of ammonium sulfate and potassium perchlorate from 2.16 to 0 *M* and from 0 to 0.16 *M*, respectively. Other conditions as in Fig. 1.

tained with this binary salt gradient is illustrated in Fig. 3D. It can be seen that the potassium perchlorate–ammonium sulfate gradient system exhibited less retentivity toward the proteins and yielded different modulation of selectivity than the potassium thiocyanate–ammonium sulfate gradient at the same eluent surface tension (*cf.* Figs. 3D and 3C).

Based on the above results, thiocyanates and perchlorates are likely to bind electrostatically to the protein molecule[23], causing different structural alterations. Indeed, replacing KSCN or $KClO_4$ by sodium chloride in the gradient former and keeping the surface tension constant (72.22 dynes/cm) did not result in improvement of resolution and selectivity; instead, the retention of all proteins in the sample increased slightly in the same way (*cf.* Figs. 3B and 3A). The salt-specific binding to protein would likely be responsible for selectivity changes in HIC of proteins when KSCN or potassium perchlorate are present in the eluent. This is in agreement with

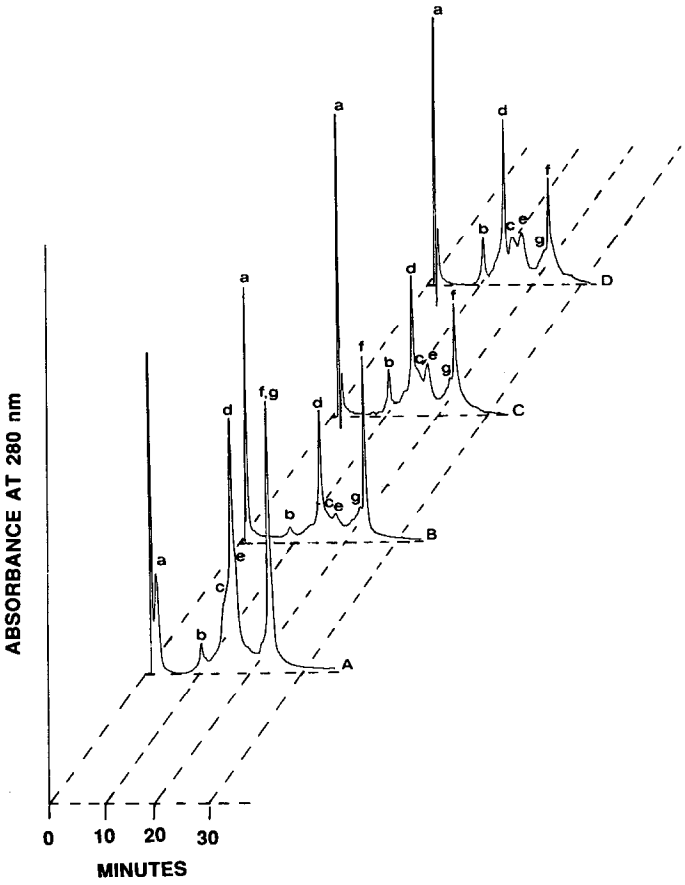

Fig. 4. Hydrophobic-interaction chromatography of proteins. (A) Ammonium sulfate gradient from 2.16 to 0 *M*; (B) ammonium sulfate and tetrabutylammonium bromide gradients from 2.16 to 0 *M* and from 0 to 10 m*M*, respectively; (C) ammonium sulfate and tetrabutylammonium bromide gradients from 2.16 to 0 *M* and from 0 to 20 m*M*, respectively; (D) ammonium sulfate and tetrabutylammonium bromide gradients from 2.16 to 0 *M* and from 0 to 40 m*M*, respectively. Other conditions as in Fig. 1.

the finding that the retention volumes of some proteins decreased while those of others increased with increasing sodium-perchlorate concentration in the eluent[2].

Binary salt gradients of decreasing ammonium sulfate concentration and increasing tetrabutylammonium (Bu_4N^+) bromide concentration (organic salt of negative molal surface tension increment) in 50 m*M* phosphate buffer (pH 6.5) were carried out. The chromatograms obtained by using 0, 10, 20 or 40 m*M* Bu_4N^+ in the gradient former are depicted in Fig. 4. Whereas cytochrome *c* was eluted in the void volume of the column in the presence of Bu_4N^+, ribonuclease A was slightly more retained at 10 and 20 m*M* than at 0 or 40 m*M* Bu_4N^+ in the eluent. Lysozyme, α-chymotrypsinogen A, and fetuin retention decreased monotonically but at different rates with increasing Bu_4N^+ concentration in the eluent. In contrast, the retention of β-lactoglobulin A first increased when going from 0 to 10 m*M* Bu_4N^+ in the gradient former and then remained unchanged as the concentration of the organic salt increased in the concentration range studied, while the retention of ovalbumin increased almost monotonically with the concentration of the organic salt in the gradient former.

The effect of Bu_4N^+ concentration on protein retention can be explained by ion-pair formation in the eluent as well as by the adsorption of such ions on the stationary phase surface. Both mechanisms can explain the general increase and decrease in retention of acidic and basic proteins, respectively, with increasing Bu_4N^+ concentration in the eluent. The exception that fetuin (an acidic protein) retention decreased with increasing Bu_4N^+ concentration can be explained by the decrease in surface tension of the eluent, since fetuin was eluted at the end of the gradient run. Ion-pair formation between Bu_4N^+ and the acidic residues of proteins would increase the hydrophobicity of the proteins and therefore their retention. On the other hand, at relatively high salt concentrations in the eluent, Bu_4N^+ ions are likely to be adsorbed on the surface of the stationary phase, thus creating a dynamically coated surface with positively charged sites. It has been shown[24] that a hydration layer deficient in salt exists on a protein surface when in contact with aqueous solutions containing high concentrations of salt. Hydrophobic interactions are of short range, allowing a close association of the protein and the stationary phase. Therefore, in the presence of positively charged sites on the hydrophobic surface, cooperative hydrophobic and coulombic interactions may take place at the contact surface area between the analyte and the stationary phase. This may explain the increase in retention for ovalbumin and β-lactoglobulin A upon adding Bu_4N^+ to the mobile phase. In contrast, the presence of adsorbed Bu_4N^+ ions on the stationary phase surface would explain the decrease in retention for basic proteins, due to weak electrostatic repulsion between species of same charges. Indeed, El Rassi and Horváth[26,27] and Kennedy *et al.*[28] have shown that ion-exchange stationary phases could be used in the HIC mode with eluents containing a high salt concentration. The elution pattern of proteins with such phases was different from that obtained by using neutral and weakly hydrophobic stationary phases, due to the interplay of hydrophobic and electrostatic interactions. Due to the specific binding of Bu_4N^+ to the proteins and/or the stationary phase, the observed retention behavior is different from that predicted by the surface tension argument of the original retention model of Horváth and coworkers[1,2] (see Introduction). Such binding may affect retention to a certain degree, thereby leading to changes in selectivity.

HIC selectivity can be further manipulated by using ternary salt gradients. The adjusted retention volumes of proteins, measured with a linear gradient of increasing both ammonium sulfate and sodium tartrate concentrations and decreasing potassium thiocyanate concentrations in the eluent are reported in Table II; gradient VIII. It is seen that the elution pattern with a ternary salt gradient is quite different from that obtained by using any of the binary salt gradients (compare gradient VIII with gradients III, IV and VII in Table II) at virtually the same eluent surface tension. Comparison of ternary gradient VIII with the binary gradient VII reveals that protein retention has increased and a reversal in the elution order of the eluite pair lysozyme and α-chymotrypsinogen A and the pair fetuin and α-chymotrypsinogen A has occurred when going from the binary to the ternary salt gradient.

The adjusted retention volumes of proteins, measured with a ternary salt gradient of decreasing ammonium sulfate and sodium citrate concentrations and increasing potassium thiocyanate concentrations in the eluent are shown in Table II; gradient X. The comparison of retention data, obtained by using the ternary gradient (X in Table II) with those measured with the binary gradient (IX in Table II) shows that the adjusted retention volumes of all the components of the mixture increased, except for ovalbumin, which decreased when using the ternary gradient instead of the binary gradient. In addition, the elution order, which was lysozyme < α-chymotrypsinogen A < fetuin with the binary salt gradient, has changed to fetuin < lysozyme < α-chymotrypsinogen A with the ternary gradient.

In conclusion, sample selectivity in HIC can be varied conveniently by holding the surface tension of the eluent constant while (i) exchanging one salt for another in single-salt gradients or (ii) varying the composition and nature of salts in binary and ternary salt gradients. Gradient elution with two or three salts is more efficient than single-salt gradients in modulating HIC selectivity. Work is continuing in this laboratory on the use of mixed aqueous salt solutions in HIC of proteins; isocratic elution is being carried out, and the results will be reported in an upcoming article.

ACKNOWLEDGEMENTS

The authors wish to thank R. W. Stout for his generous gift of Zorbax silica gel. This work was supported by grant No. HN9-004 from the Oklahoma Center for the Advancement of Science and Technology (Oklahoma Health Research Program) and by the College of Arts and Sciences, Dean Incentive Grant Program at Oklahoma State University.

REFERENCES

1 W. R. Melander and Cs. Horváth, *Arch. Biochem. Biophys.*, 183 (1977) 200.
2 W. R. Melander, D. Corradini and Cs. Horváth, *J. Chromatogr.*, 317 (1984) 67.
3 O. Sinanoğlu and S. Abdulnur, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 24 (1965) s-12.
4 T. Halicioğlu and O. Sinanoğlu, *Ann. N.Y. Acad. Sci.*, 158 (1969) 308.
5 J. L. Fausnaugh and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 131.
6 Z. El Rassi and Cs. Horváth, *J. Liq. Chromatogr.*, 9 (1986) 3245.
7 A. Katti, Y.-F. Maa and Cs. Horváth, *Chromatographia*, 24 (1987) 646.
8 J. L. Fausnaugh, L. A. Kennedy and F. E. Regnier, *J. Chromatogr.*, 317 (1984) 141.
9 M. N. Schmuck, M. P. Nowlan and K. M. Gooding, *J. Chromatogr.*, 371 (1986) 55.
10 N. T. Miller and B. L. Karger, *J. Chromatogr.*, 326 (1985) 45.

11 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 333 (1985) 202.
12 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 298 (1984) 407.
13 H. Engelhardt and U. Schon, *J. Liq. Chromatogr.*, 9 (1986) 3225.
14 S. Hjerten, K. Yao, K.-O. Eriksson and B. Johansson, *J. Chromatogr.*, 359 (1986) 99.
15 S. C. Goheen and S. C. Engelhorn, *J. Chromatogr.*, 317 (1984) 55.
16 M. L. Heinitz, L. Kennedy, W. Kopaciewicz and F. E. Regnier, *J. Chromatogr.*, 443 (1988) 173.
17 D. B. Wetlaufer and M. R. Koenigbauer, *J. Chromatogr.*, 359 (1986) 55.
18 S.-L. Wu, K. Benedek and B. L. Karger, *J. Chromatogr.*, 359 (1986) 3.
19 A. J. Alpert, *J. Chromatogr.*, 359 (1986) 85.
20 D. L. Gooding, M. N. Schmuck and K. M. Gooding, *J. Chromatogr.*, 296 (1984) 141.
21 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 360 (1986) 260.
22 J.-P. Chang, Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 319 (1985) 396.
23 T. Arakawa and S. N. Timasheff, *Biochemistry*, 21 (1982) 6545.
24 T. Arakawa and S. N. Timasheff, *Biochemistry*, 23 (1984) 5912.
25 T. Arakawa and S. N. Timasheff, *Methods Enzymol.*, 114 (1985) 49.
26 Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 326 (1985) 79.
27 Cs. Horváth and Z. El Rassi, *Chromatography Forum*, 1 (1986) 49.
28 L. A. Kennedy, W. Kopaciewcz and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 73.