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# GRADIENT AND ISOCRATIC HIGH-PERFORMANCE HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS ON AGAROSE COLUMNS

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## SUMMARY

Columns of pentyl and octyl agarose (6–8 cm × 6 mm I.D., bead size 5–7 μm) have been used successfully for high-performance hydrophobic interaction chromatography of both model proteins and the proteins in human serum. Interestingly, these columns can be eluted not only with a negative salt gradient but also isocratically. The efficiency of the columns is high (around 15 000 plates per metre for cytochrome *c* in isocratic elution). In the range pH 2.5–11.5 the capacity factor changed drastically for some, but not all, proteins (particularly below pH 5.5 and above 8.5), which means that pH is an important separation parameter in the optimization of resolution in hydrophobic interaction chromatography. The residence time of the proteins on the column has no observable influence on the appearance of the chromatograms. Some experimental conditions which are required for high resolution in isocratic elution are discussed.

## INTRODUCTION

Classical hydrophobic interaction chromatography (HIC) on neutral, amphiphilic agarose gel derivatives, as developed in this laboratory[1], rapidly became a routine separation method for proteins after the gels became commercially available (Octyl and Phenyl Sepharose®). We have also shown that agarose can be used as a matrix for different forms of high-performance liquid chromatography (HPLC)[2–11], including HIC[12]. The present paper is a more extensive account of this latter HPLC technique. For matrices other than agarose used in high-performance hydrophobic interaction chromatography, see refs. 13–21.

## MATERIALS

The agarose (EEO-0.17) was from Reactifs IBF (Villeneuve la Garenne, France), γ-glycidoxypropyltrimethoxysilane (trade-name, Dow Corning Z-6040 sil-

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ane) from Dow Corning (Midland, MI, U.S.A.) and $SnCl_4 \cdot 5H_2O$ from Malinckrodt (St. Louis, MO, U.S.A.). Cytochrome *c*, lysozyme, egg trypsin inhibitor and soy bean trypsin inhibitor were purchased from Sigma (St. Louis, MO, U.S.A.), ovalbumin, ribonuclease A and Octyl Sepharose CL 4B® from Pharmacia Fine Chemicals (Uppsala, Sweden). Human serum albumin and transferrin were gifts from Kabi Vitrum (Stockholm, Sweden) and enolase from Dr. Göran Pettersson (this Institute).

## INSTRUMENTATION

The chromatographic system, including 2150 HPLC pumps, 2152 HPLC controllers, 2138 and 2158 UV detectors, a 2220 recording integrator and a 2210 recorder, was from LKB (Bromma, Sweden). Loop injectors were purchased from Rheodyne (Berkeley, CA, U.S.A.).

## METHODS

All experiments were performed on 12% agarose beads, prepared as described previously[22] and fractionated by elutriation in water. A fraction containing beads of diameters between 5 and 7 $\mu$m was collected and used throughout this study. The cross-linking of agarose gel beads will be described elsewhere. The attachment of alkyl ligands was performed as described in ref. 23 via $\gamma$-glycidoxypropyltrimethoxysilane with $SnCl_4$ as catalyst.

The Plexiglas column tubes had an inner diameter of 0.6 cm and were packed to a height of 6–8 cm. The absorbance measurements were made at 280 nm.

The buffer concentration was 0.05 *M* for all pH values. For pH 2.5 we used a glycine–HCl buffer; for pH 4.0 and 5.3, sodium acetate; for pH 7.0, sodium phosphate; for pH 8.5, Tris–HCl; and for pH 11.5, sodium phosphate. In all of the experiments described, ammonium sulphate was used to induce hydrophobic interaction except at high pH values, where we employed sodium sulphate since the ammonium ion is converted into ammonia in strongly alkaline media. At pH 8.5 we used both sodium sulphate and ammonium sulphate.

## EXPERIMENTAL AND RESULTS

### *Protein capacity*

The effective ligand densities of pentyl and octyl agarose and the commercial Octyl Sepharose CL 4B were measured indirectly by determination of the protein

TABLE I

PROTEIN CAPACITY FOR PENTYL AGAROSE, OCTYL AGAROSE AND OCTYL SEPHAROSE CL-4B

| | *Protein capacity (mg lysozyme adsorbed per g dried gel)* |
|---|---|
| Pentyl agarose | 1.7 |
| Octyl agarose | 3.7 |
| Octyl Sepharose CL-4B | 6.3 |

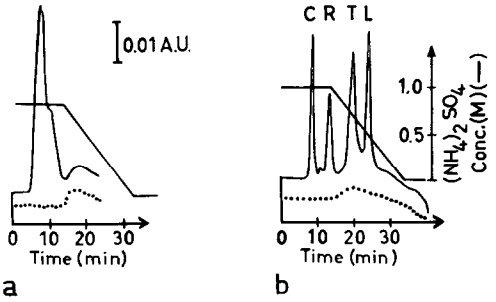


Fig. 1. A comparison of the hydrophobicity of cross-linked agarose beads without (a) and with (b) octyl ligands. Sample: cytochrome *c* (C), ribonuclease A (R), transferrin (T) and lysozyme (L). The dotted line represents the recorder tracing in a control experiment without sample. The full line indicates the shape of the gradient. Fig. 1a shows that the hydrophobicity of the agarose matrix has a negligible influence on the protein separations obtained on octyl agarose (Fig. 1b).

capacity, *i.e.*, the amount of lysozyme adsorbed on a 1.2 cm × 6 mm I.D. column, equilibrated with 0.05 *M* sodium phosphate buffer (pH 7.0), containing 1.0 *M* ammonium sulphate. The equilibrated column was saturated with lysozyme, dissolved in the buffer used for the equilibration. The excess of lysozyme was removed with the same buffer. Desorption of lysozyme was achieved by 0.05 *M* sodium phosphate (pH 7.0). The protein amounts in the eluate were calculated from measurements of volume and absorption at 280 nm. The protein capacities calculated are listed in Table I.

*Does the cross-linked non-derivatized agarose matrix interact hydrophobically with proteins?*

A column with an inner diameter of 6 mm was packed with cross-linked agarose beads to a height of 6 cm. After equilibration of the bed with 1.0 *M* ammonium

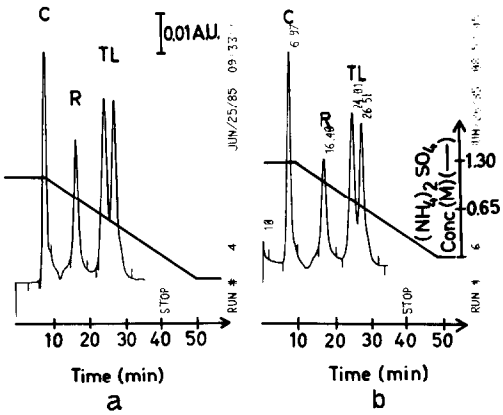


Fig. 2. Chromatography showing that the adsorption is not time-dependent. The sample contained 30–70 μg of each of the proteins: cytochrome *c* (C), ribonuclease (R), transferrin (T) and lysozyme (L). Sample volume: 20 μl. Bed dimensions: 5.5 cm × 6 mm I.D. Flow-rate: 0.2 ml/min. Gradient: from 1.3 to 0 *M* ammonium sulphate in 0.05 *M* Tris–HCl in 40 min. The elution was started immediately after the application of the sample (a) and 16 h after that (b). The great similarity between the chromatograms indicates that the residence time has no observable influence on the adsorption.

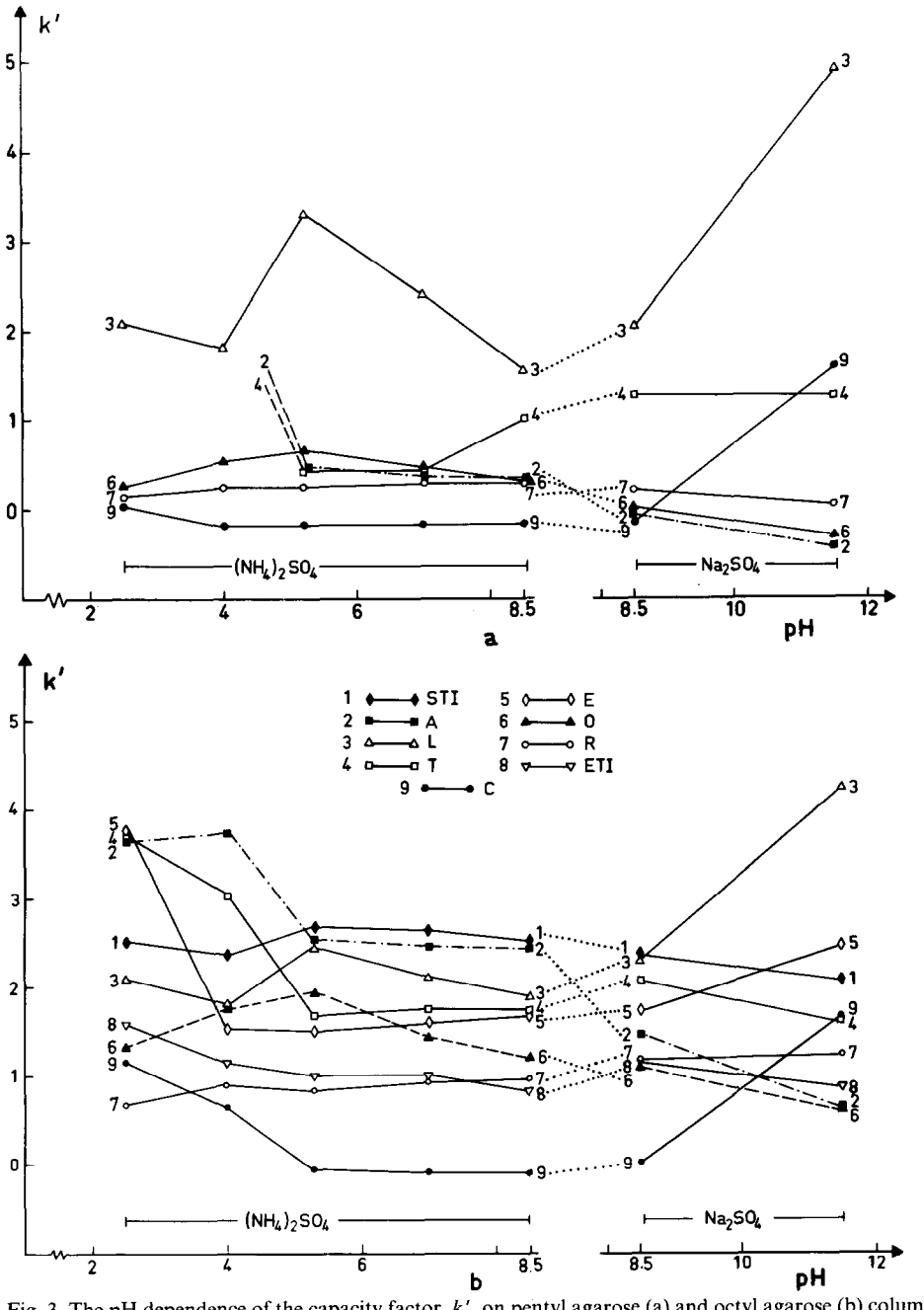


Fig. 3. The pH dependence of the capacity factor, $k'$, on pentyl agarose (a) and octyl agarose (b) columns. Flow-rate: 0.2 ml/min. For the pentyl agarose column (a) isocratic elution was used (1.04 *M* ammonium sulphate for pH 2.5–8.5 and 0.78 *M* sodium sulphate for pH 8.5 and 11.5). For the octyl agarose column (b) gradient elution for 40 min was employed (for pH 2.5–8.5 a linear descending gradient from 1.3 to 0 *M* ammonium sulphate and for pH 8.5 and 11.5 a linear descending gradient from 1.0 to 0 *M* sodium sulphate). The model proteins used are listed in Fig. 3b, where STI = soy bean trypsin inhibitor, A = human serum albumin, L = lysozyme, T = transferrin, E = enolase, O = ovalbumin, R = ribonuclease, ETI = egg trypsin inhibitor and C = cytochrome *c*.

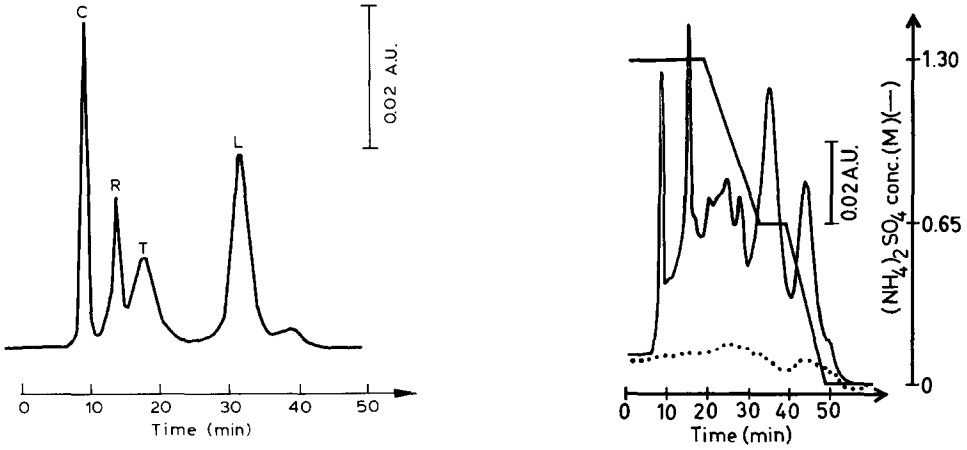


Fig. 4. Isocratic separation of model proteins by high-performance HIC. on pentyl agarose. The sample contained 50 μg of each of the proteins cytochrome *c* (C), ribonuclease A (R), transferrin (T) and lysozyme (L). Sample volume: 20 μl. Bed dimensions: 8 cm × 6 mm I.D. Flow-rate: 0.2 ml/min. Eluent: 0.05 *M* sodium phosphate (pH 7.0), containing 1.04 *M* ammonium sulphate. It should be noted that the resolution is high, in spite of the shortness of the column.

Fig. 5. Gradient separation of human serum by high-performance HIC on pentyl agarose. Sample: 20 μl of serum. Bed dimensions: 6 cm × 6 mm I.D. Flow-rate: 0.2 ml/min. Gradient: from 1.3 to 0 *M* ammonium sulphate in 0.05 *M* sodium phosphate (pH 7.0).

sulphate in 0.05 *M* sodium phosphate buffer (pH 7.0), 20 μl of a mixture containing 30–70 μg of each of the proteins cytochrome *c* (C), ribonuclease A (R), transferrin (T) and lysozyme (L) was applied. Isocratic elution for 5 min with the same buffered ammonium sulphate solution was followed by elution for 20 min with a linear negative salt gradient, formed from this solution and the phosphate buffer alone. The flow-rate was 0.2 ml/min. This blank experiment gave the chromatogram shown in Fig. 1a. The experiment was then repeated with the difference that the cross-linked agarose beads were derivatized with octyl groups (Fig. 1b). Fig. 1a indicates that cross-linked agarose (blank gel) exhibits no hydrophobic interaction. The separations obtained on octyl agarose (Fig. 1b) are therefore caused chiefly by hydrophobic interactions.

*Effect of the chromatographic residence time on the separation pattern*

An octyl agarose column, 6 cm × 6 mm I.D. was equilibrated with 0.05 *M* sodium phosphate buffer (pH 7.0), containing 1.3 *M* ammonium sulphate. The elution of a sample consisting of 30–70 μg of each of the proteins cytochrome *c*, ribonuclease, transferrin and lysozyme was performed by a 40-min gradient, formed from this solution and 0.05 *M* sodium phosphate (pH 7.0). A similar experiment with the same sample was then performed on the same column, but after application of the sample, about 0.5 ml of the starting buffer were pumped into the column (the sample was concentrated in a thin zone at the top of the column, as visualized with cytochrome *c*). After a residence time of 16 h on the column the sample was eluted by the above gradient. The two chromatograms (Fig. 2) obtained were very similar,

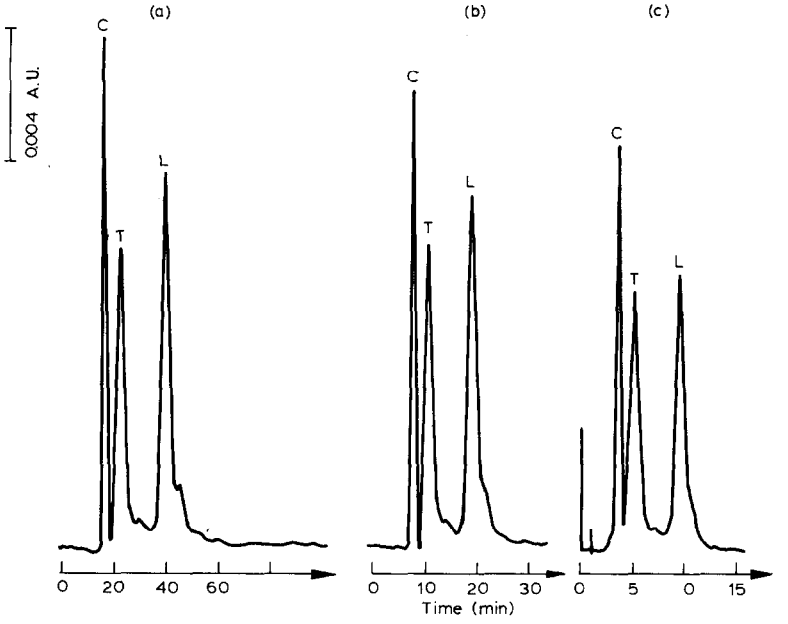


Fig. 6. Isocratic separation of model proteins by high-performance HIC on octyl agarose at different flow-rates. The sample contained 30–70 μg of each of the proteins cytochrome *c* (C), transferrin (T) and lysozyme (L). Sample volume: 20 μl. Eluent: 0.78 *M* ammonium sulphate in 0.05 *M* sodium phosphate (pH 7.0). Bed dimensions: 8.5 cm × 6 mm I.D. Flow-rates: 0.1 (a), 0.2 (b) and 0.4 (c) ml/min. It should be noted that the resolution is high in spite of the shortness of the column.

indicating that the 16-h adsorption of the proteins on the column did not affect their interactions with the matrix in a chromatographically significant way.

*The capacity factor as a function of the eluent pH*

Model proteins were subjected to high-performance hydrophobic interaction chromatography on pentyl agarose at the following pH values: 2.5, 4.0, 5.3, 7.0, 8.5

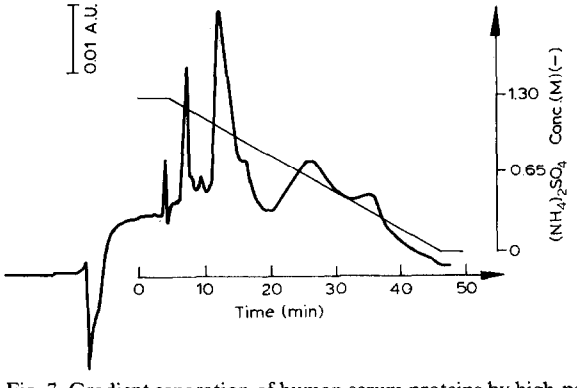


Fig. 7. Gradient separation of human serum proteins by high-performance HIC on octyl agarose. Sample: 20 μl of serum. Bed dimensions: 6 cm × 6 mm I.D. Flow-rate: 0.4 ml/min. Gradient: from 1.3 to 0 *M* ammonium sulphate in 0.05 *M* sodium phosphate (pH 7.0) in 20 min.

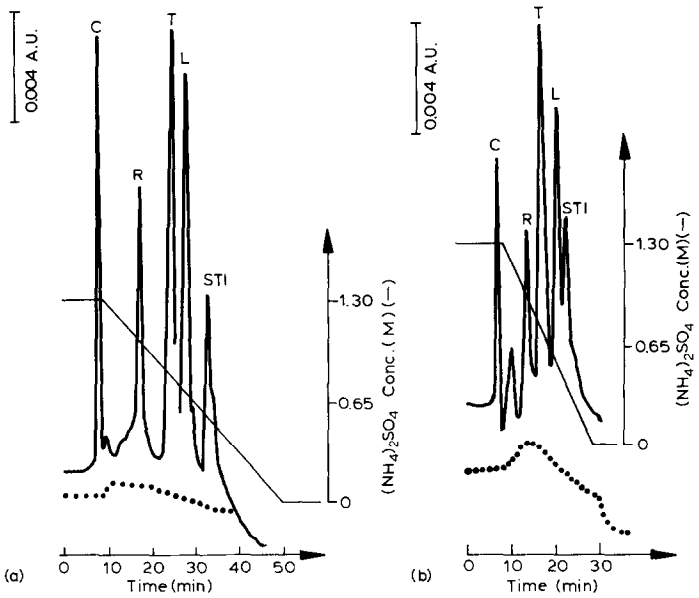


Fig. 8. Gradient separation of model proteins by high-performance HIC on octyl agarose. The sample contained 30–70 μg of each of the proteins cytochrome *c* (C), ribonuclease A (R), transferrin (T), lysozyme (L) and soy bean trypsin inhibitor (STI). Sample volume: 20 μl. Bed dimensions: 6 cm × 6 mm I.D. Flow-rate: 0.2 ml/min. A linear gradient from 1.3 *M* ammonium sulphate in 0.05 *M* sodium phosphate (pH 7.0) to buffer alone was complete either in 40 min (a) or in 20 min (b).

and 11.5. (The buffers used are described in Methods.) The proteins were applied separately, and for each experiment (isocratic elution) the capacity factor, $k'$, of the protein was determined and plotted against pH (Fig. 3a). A similar experiment was also performed on octyl agarose, with elution by a negative salt gradient (Fig. 3b).

*Applications*

Hydrophobic interaction chromatography of some model proteins on pentyl agarose is shown in Fig. 4 (isocratic elution). The same column was also utilized for a fractionation of serum proteins by gradient elution (Fig. 5). The above experiments were repeated with the principal difference that an octyl agarose column was employed (Figs. 6 and 7). In addition, this column was employed for a gradient elution of model proteins (Fig. 8).

## DISCUSSION

The agarose beads used in this study were designed to have the following three properties, which are important for high resolution, particularly in isocratic runs.

*(a) Small diameter.* In ideal gradient elution the solutes to be separated (for instance proteins) migrate down the column with continuously decreasing adsorption, which should have a finite value or approach zero when they leave the column. The solute zones are thus displaced continuously during their migration down the column, which provides zone sharpening. In this ideal case, narrow zones can there-

fore be obtained even with relatively large beads. However, if the desorption is complete before the solutes leave the column, the bead size is of greater importance, since the solute zones then migrate through part of the column without zone sharpening and therefore broaden. In the extreme case, when the solutes migrate with constant adsorption and therefore constant velocity along the whole column, *i.e.*, under conditions of isocratic elution, it is of utmost importance to reduce the bead diameter in order to obtain narrow zones. For the reasons discussed, we used beads of diameters as small as 5–7 $\mu$m.

*(b) Little non-specific interaction.* In order to achieve true hydrophobic interaction chromatography, the ligands must be uncharged. Otherwise the retention will be affected by both hydrophobic and electrostatic interactions. These dual interactions may either facilitate or hinder the separation of particular proteins, but may in some cases make it impossible to desorb a protein[24,25], since the decrease in salt concentration required to decrease the hydrophobic interaction will increase the electrostatic one (a prerequisite for desorption is that both interactions are virtually nullified). Therefore we have employed a coupling method that gives uncharged ligands[23].

The agarose matrix used in this study exhibits no hydrophobic interactions, even at a salt concentration as high as 1.0 *M* ammonium sulphate (see Fig. 1a).

*(c) Low density and hydrophobicity of the ligands.* It is well known that isocratic elution of proteins rarely gives high resolution. This is true for all kinds of adsorbents (but not for molecular-sieving gels, where the solutes are partitioned, not adsorbed). However, amino acids can be well separated on a number of adsorbents under isocratic conditions. Since these compounds are the constituents of proteins and thus bind to the adsorbents by the same forces as proteins, it is evident that the molecular size of the solutes to be separated, *i.e.*, the number of adsorption sites in the solutes, is a parameter that has a great influence on the resolution obtained in isocratic elution. A prerequisite for successful isocratic separation of proteins and other macromolecules, such as nucleic acids and polysaccharides, is, accordingly, that the number of bonds between the macromolecules and the adsorbent be relatively low. The pronounced multi-point attachment which is characteristic of macromolecules under most commonly used chromatographic conditions[26,27] is therefore probably the cause of the difficulties in separating biopolymers isocratically. A means of decreasing the number of bonds (the adsorption) between the biopolymers and the adsorbent is to decrease the ligand densities, as pointed out previously[28].

The ligand densities of the columns used in the experiments presented herein are given in Table I in terms of protein capacity. These values are considerably lower than those of the silica-based amphiphilic columns employed by Fausnaugh *et al.*[20], who reported that "although proteins may be eluted from the HIC columns isocratically, the peaks were very broad and the resolutions were very poor". The chromatograms in Figs. 4 and 6 show that the agarose beads prepared for this investigation can be utilized with advantage for isocratic elution, the probable reason being their relatively low ligand density (Table I), the absence of non-specific hydrophobic interaction (Fig. 1) and their small diameter (5–7 $\mu$m), three properties which favour high resolution in isocratic elution, as discussed above. A fourth favourable property is that they have linear adsorption isotherms (probably related to the first and the second properties mentioned), as is evident from the symmetry of the peaks in the

chromatograms in Figs. 4 and 6. For most adsorbents, however, the isotherms for macromolecules are strongly curved, causing a strong tailing of the solute zones in isocratic elution.

It is well known that the strength of adsorption is a function not only of the ligand density but also of the hydrophobicity of the ligand[28,29]. To obtain weak hydrophobic interactions, facilitating the desorption process, and thus isocratic elution, we have therefore used relatively short alkyl chains (pentyl and octyl groups). Low ligand hydrophobicity can be considered a fifth property of the beads that favours isocratic elution.

In isocratic elution the resolution increases with the length of the column. It is therefore surprising that the separations presented in Figs. 4 and 6 are so good in spite of the shortness of the columns (around 8 cm).

From these considerations, one can understand that even at low ligand density a relatively strong non-specific hydrophobic interaction (see above) can exclude the possibility of attaining good resolution upon isocratic elution. The agarose columns used in this investigation have not shown such a disturbing interaction (Fig. 1).

The possibility of using isocratic elution for HIC columns will make the technique more versatile, and therefore deserves further investigation.

*The efficiency of the columns*

The plate numbers for cytochrome *c*, ribonuclease and lysozyme in isocratic elution on pentyl agarose shown in Fig. 4 (flow-rate, 0.2 ml/min) were calculated as 10 400, 12 600 and 12 000 per metre, respectively; the plate number for transferrin was not calculated, as it was very heterogeneous when tested by isoelectric focusing in the high-performance electrophoresis apparatus[30]. For cytochrome *c* and lysozyme in Fig. 6a (octyl agarose) the following plate numbers were otained (flow-rate, 0.1 ml/min): 15 600 and 9600 per metre, respectively. When the flow-rate was increased to 0.2 (Fig. 6b) and 0.4 ml/min (Fig. 6c), the plate numbers were 15 300 and 8100 and 11 000 and 7900 per metre, respectively. These relatively high plate numbers indicate the high efficiency of the columns, which is also evident from their small flow dependence (Fig. 6). Agarose columns used for molecular sieving have given plate numbers of around 25 000 per metre for cytochrome *c*[6].

*The time dependence of the adsorption*

Hydrophobic interaction chromatography is a milder method than reversed-phase chromatography, since no denaturing organic solvents are used. This is reflected by the observation that the appearance of the chromatogram is affected by the residence time of the proteins on the columns in the latter[31] but not in the former method, at least not with the agarose columns used in this investigation (see Fig. 2).

*The capacity factor as a function of pH*

It has been shown that the pH of the eluent can be used to modulate protein separations in classical low-pressure HIC[32]. The effect of pH on protein retention in the high-performance mode on silica beads has also been studied[19], but for known reasons the highest pH used was 8. In our experiments, we have found that the retention of proteins changes more drastically at pH values above 8.5 and/or below 5 than in the range pH 5–8.5. Since the magnitude of these alterations in the retention

is different for different proteins, the eluent pH is an important separation factor that should be utilized to obtain optimum resolution. Fig. 3a and b show that one should routinely test three different values in HIC: one in the range pH 6–8, one in the range 9–11 and one in the range 2.5–4. Fig. 3 also shows that sodium ions and ammonium ions affect the capacity factors differently for several proteins (see the points for pH 8.5). For some proteins the connecting lines (dotted) for these points cross each other, indicating that the order of elution of these proteins is reversed when ammonium sulphate is used instead of sodium sulphate. Accordingly, not only the pH but also different salts should be used to optimize the separation.

No relationship between the rate of change of the capacity factor, $k'$, and the isoelectric points was observed, although the two basic proteins lysozyme and cytochrome *c* were retarded to a larger degree at high pH values than were the other proteins tested (Fig. 3).

*The pH stability of the ligand bond*

No significant leakage at pH 10 of octyl groups attached to agarose by γ-glycidoxypropyltrimethoxysilane (the coupling agent used in this investigation) was observed over a period of two months[23]. This pH stability is of importance, since it is at alkaline pH that large alterations in capacity factors occur, which can be utilized to optimize a separation (see above).

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