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HYDROPHOBIC CHROMATOGRAPHY ON HOMOLOGOUS SERIES OF ALKYLAGAROSES

A COMPARISON OF CHARGED AND ELECTRICALLY NEUTRAL COLUMN MATERIALS

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SUMMARY

The relative contribution of hydrophobic and ionic interactions during chromatography of proteins on homologous series of hydrocarbon-coated agaroses (obtained by the coupling of alkylamines to CNBr-activated agarose) was assessed by comparing these columns, which contain some positive charges, with two companion column series devoid of charge (prepared either by acetylation of the above series or by the glycidyl-ether coupling procedure). Using a variety of pure proteins as well as a protein mixture extracted from muscle, it is shown that columns with identical ligand densities have (qualitatively and quantitatively) similar adsorption and discrimination properties whether they are charged or not, leading us to conclude that hydrophobic (rather than ionic) interactions play the main role in the mechanism of chromatography on such columns.

INTRODUCTION

It is now commonly accepted that many proteins, in their native, biologically functional conformation, possess hydrophobic "patches" or "pockets" on their surface[1]. In the case of membranal proteins, these hydrophobic patches may extend over most of the surface of the molecule and play a major role in targeting the protein to the membrane. On the other hand, the relatively small hydrophobic "patches" on the surface of water-soluble proteins may, at least in some cases, have a distinct biological assignment in protein–protein recognition, *e.g.* in the assembly of multi-enzyme complexes or intracellular organelles. Owing to the occurrence of such hydrophobic patches, it is possible to retard and retain proteins on column materials which offer immobilized hydrophobic ligands[2–7].

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Different proteins vary in the hydrophobic patches or pockets on their surface. They may vary in the number of such patches per molecule as well as in their size, shape and lipophilicity. These variations would then be reflected in the relative affinities of these proteins for a given hydrocarbon and also in the stoichiometry of binding. With the introduction of homologous series of hydrocarbon-coated agaroses[3,4], it became possible to take advantage of these differences and to establish a systematic approach to the resolution, purification and probing of proteins[8–11] and cells[12,13], based on the hydrophobic nature of their surface.

When the name "hydrophobic chromatography" was proposed[4] to describe this technique it was emphasized that, *a priori*, several types of interaction might be involved simultaneously in the retention of proteins by these columns. Special mention was made of ionic interactions, because it was already known[14,15] that upon activation of agarose with CNBr and coupling with primary amines in general, positive charges are introduced into the column materials, a fact which was confirmed by subsequent studies[16]. Nevertheless, it seemed justifiable to conclude[4] that "hydrophobic interactions must contribute dominantly to the retention and discrimination power of these columns". This conclusion was based on several pieces of evidence, the major one being that the homologous series used in our studies were identical in all structural respects tested (including their ligand density and charge density), yet they did differ in their capacity to retain proteins —passing from no retention through retardation and reversible binding up to very tight binding, with increasing hydrocarbon chain length. Other workers in this field argued[5,17,18] that electrostatic interactions play an important (or even a decisive) role in the retention of proteins by Seph-C_n columns*, when prepared by the CNBr procedure. To support their argument they cited experimental evidence to the effect that it is often possible to achieve elution of proteins from such columns with increasing salt concentration[17], that some basic proteins (*e.g.* lysozyme), which are positively charged at neutral pH, do not bind to Seph-C_1, Seph-C_4 and Seph-C_8 in the above series[5], and finally that on acetylation of Seph-C_4 or Seph-C_6 (which cancels their positive charge) ovalbumin and α-lactalbumin do not bind to these columns any more[18].

This paper attempts to assess the relative importance of hydrophobic *vs.* ionic interactions during chromatography on homologous series of hydrocarbon-coated agaroses by using structurally comparable charged and non-charged series of columns and by following the adsorption profiles of some twenty different proteins.

MATERIALS AND METHODS

Chemicals

Sepharose-4B was obtained from Pharmacia (Uppsala, Sweden), BF_3 from Eastman-Kodak (Rochester, NY, U.S.A.), and epichlorohydrin and CNBr from Fluka (Buchs, Switzerland). *n*-Alkylamines (butyl-, hexyl-, octyl-, decyl-, and dodecylamine) were obtained from Merck-Schuchardt (Hohenbrum bei München,G.F.R.). *n*-Alkyl alcohols (butyl, hexyl, octyl, decyl, dodecyl alcohol) were obtained from BDH (Poole, Great Britain). All other chemicals were best available grade from commercial sources.

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* Seph-C_n is the abbreviation for Sepharose 4B on to which normal-hydrocarbon chains with *n* carbon atoms have been covalently attached.

Radiochemicals

n-[1-^{14}C]Dodecyl alcohol (specific activity 30 mCi/mmole) and *n*-[1-^{14}C]-dodecylamine acetate (specific activity 1 mCi/mmole) were both from The Radiochemical Centre (Amersham, Great Britain).

Synthesis of Seph-C_n by the CNBr procedure (Type I)

The column materials in this series were prepared as described earlier[3,8], except for the fact that the coupling step was carried out in dioxane–water (95:5, v/v), allowing the reaction to proceed for 20 h (pH 9.0) at 22°C. The column materials were then washed successively with 2–3 volumes of each of the following dioxane–water mixtures (v/v): 95:5; 80:20; 60:40; 40:60; 20:80. The resulting gel was then transferred into 0.1 *M* $NaHCO_3$, pH 9.5, shaken for 1 h at room temperature, then washed with *ca.* 20 volumes of water and suspended in the desired buffer. In the case of Seph-C_{12} (Type I), the reaction was carried out with *n*-[1-^{14}C]dodecylamine with a specific activity of 0.65 mCi/mole.

Acetylation of Seph-C_n (Type I) to obtain the "Type I, Acet" series

Acetylation of Seph-C_n columns was performed with acetic anhydride using the procedure described by Wilchek and Miron[18].

Synthesis of Seph-C_n (Type II) by binding of alkylglycidyl ethers to agarose

The synthesis of glycidyl ethers from the appropriate alcohols was carried out according to Ulbrich *et al.*[19], and their coupling to agarose (Sepharose 4B) as described by Hjertén *et al.*[20]. In the case of Seph-C_{12} (Type II), the coupling was carried out with *n*-[1-^{14}C]dodecyl glycidyl ether with a specific activity of 0.89 mCi/mole.

Dry weight determinations of the column materials

The column material to be tested was washed with *ca.* 20 volumes of deionized water and then placed on a Buchner funnel to drain off excess water until the very first signs of dryness appeared. Three 1-g samples of each gel were weighed and dried *in vacuo* at 120°C to constant weight, to establish the dry weight of a given column material.

Determination of the ligand density of the column materials

Using radioactively labelled ligands. Samples of column materials (0.3 g of settled gel) were dissolved in 1 ml of 6 *N* HCl and 10 ml of triton–toluene scintillator were added. The mixture was shaken until a clear solution was formed, then counted in a Packard Tricarb scintillation counter. The scintillation liquid was composed of 0.1 g of 1,4-bis-[2-(5-phenyl oxazolyl)]benzene, 8 g of 2,5-diphenyloxazole, 666 ml of toluene and 333 ml of Triton X-100. The efficiency of radioactivity counting in our experimental system was determined by counting several identical samples of *n*-[1-^{14}C]dodecyl alcohol in the scintillator, both alone and in the presence of samples (0.3 g of settled gel) of the column materials dissolved in 1 ml of 6 *N* HCl as described above. The counting efficiency was found to be 72%, and the experimental values were corrected accordingly.

Determination of ligand densities by nuclear magnetic resonance (NMR). The method used for the NMR determination of ligand densities in gels obtained by the

epoxy-coupling (Type II) was that described by Rosengren *et al.*[21]. The spectra were taken in [$^2H_6$]dimethyl sulphoxide using either an HFX-10 Bruker 90 MHz NMR spectrometer connected to a 1080-Nicolet computer (FT mode) or a Varian T-60 NMR spectrometer (CW mode). Tetramethylsilane was used as an internal reference. This method was also adapted to gels obtained by the CNBr coupling method after appropriate modification of the equation for the calculation of the molar ratios (MR) of alkyl chains to galactose units.

According to Rosengren *et al.*[21]:

$$MR_{\text{Type II}} = \frac{\text{moles of alkyl chains}}{\text{moles of galactose units}} = \frac{\dfrac{B + C}{2(n - 2) + 3}}{\dfrac{A - \dfrac{7(B + C)}{2(n - 2) + 3}}{7}}$$

(For the definition of A, B, C and n see ref. 21 and Fig. 1.)

Based on the structural differences between the Type I and Type II column materials:

$$MR_{\text{Type I}} = \frac{\text{moles of alkyl chains}}{\text{moles of galactose units}} = \frac{\dfrac{B + C}{2(n - 2) + 3}}{\dfrac{A - \dfrac{2(B + C)}{2(n - 2) + 3}}{7}}$$

For both types of column material the ligand densities were calculated as follows:

$$\text{ligand density} = \frac{\text{moles of alkyl chains}}{\text{grams of wet gel}} = \left(\frac{\text{dry weight of 1 g of gel}}{(MR \times MW) + 153}\right)(MR)$$

(where MW stands for the molecular weight of the ligand and 153 represents the average molecular weight of the carbohydrate residues in the agarose).

As seen in Table I, the ligand density values determined on samples of the same gel by ^{14}C incorporation and by NMR were in good agreement, confirming the findings of Rosengren *et al.*[21] for the Type II column materials, and extending the use of this method to the Type I column materials. The NMR method was therefore routinely used when radioactive ligands were not available.

Determination of charge densities of the column materials

Column materials to be titrated were thoroughly washed with 1 *M* KCl solution, filtered and then suspended in the same KCl solution —so that 3 ml of suspension contained 0.5 ml (settled volume) of column material.

Potentiometric titrations were carried out with a Radiometer instrument (TTTIC) equipped with a microtitration kit (TTA 31), a temperature-controlled compartment (pHA 924) and an automatic recorder (SBR2C). The titration chamber was kept under nitrogen during the titration, and thermostated at 25°C. The pH was

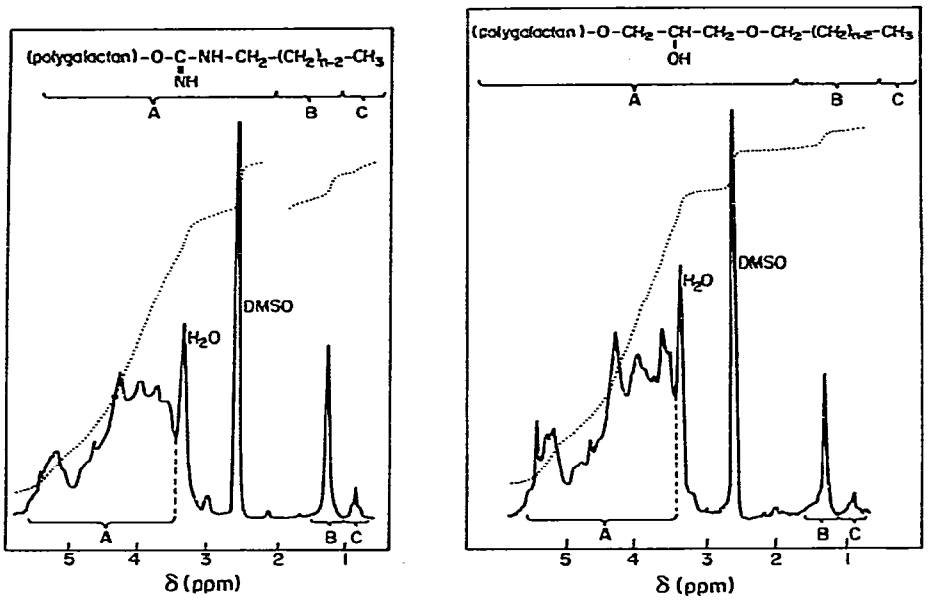


Fig. 1. NMR (90 MHz) spectrum of decylagarose prepared by the CNBr coupling procedure (Seph-C_{10} Type I, left panel) or by the glycidyl ether coupling procedure (Seph-C_{10} Type II, right panel). The ligand densities of the column materials used in this experiment were calculated from these spectra and found to be 13 (Type I) and 8.5 (Type II) μmole per gram of wet gel (*cf.* Materials and methods).

adjusted to 6.5 (with 0.2 *N* HCl), and then the automatic titration was carried out with 50 m*M* KOH up to pH 11.5. Blanks of unmodified Sepharose 4B (from the same batch) were similarly treated and titrated. The results shown in the titration curve figures represent the difference between the titration curves obtained with the tested column materials, and those obtained with the blanks of the Sepharose 4B.

Slab gel electrophoresis

Polyacrylamide gel electrophoresis[22] was carried out using a linear polyacryl-

TABLE I

COMPARISON OF LIGAND DENSITY VALUES DETERMINED BY THE USE OF RADIOACTIVELY LABELLED LIGANDS AND BY NMR

Gel	*Ligand density (μmole/g wet gel)*	
	By ^{14}C-incorporation	*By NMR*
Seph-C_{12} (type I)	12	12
	10.3	9.8
	3.5	3.0
Seph-C_{12} (Type II)	22.0	21.6
	5.5	6.3
	3.7	3.7
	1.2	1.9

amide gradient gel (7–20%) and a 25 m*M* Tris-glycine buffer (pH 8.6) containing 0.1% of sodium dodecylsulphate. The gels were stained with Coomassie blue (0.25%) in a solvent composed of methanol–14% acetic acid (50:50). Destaining was performed with the same solvent. The densitometric scans of the destained gels were performed with a Gilford spectrophotometer (Model 240) equipped with a gel scanning device (Model 2410-S).

Preparation of crude muscle extract

Minced rabbit skeletal muscle (100 g) was suspended in 250 ml of deionized water. Extraction was allowed to proceed for 15 min at 22°C. The suspension was filtered through cheese-cloth, centrifuged (40 min at 2000 *g*), filtered again through glass wool, then dialyzed three times against 2 l of the buffer to be used during passage through the column.

Proteins

Glycogen phosphorylase *b* (from rabbit muscle) was prepared by the method of Fischer *et al.*[23], with the modifications introduced by Krebs *et al.*[24]. Prior to use, the enzyme was dissolved in the appropriate buffer and freed from AMP by passage through a charcoal–Sephadex column. The other proteins used were obtained from Sigma (St. Louis, MO, U.S.A.).

Protein concentrations

Protein concentrations were determined either by monitoring the absorbance at 280 nm or by the method of Lowry *et al.*[25]. For the determination of myoglobin (from sperm whale) the protein was first treated with NaCN and a trace of $K_3Fe(CN)_6$. Its concentration (in the cyanomet form) was then measured at 540 nm. Appropriate calibration curves (with known amounts of the protein to be determined) were used in each case.

Protein binding capacity of the column materials

The protein-binding capacity of the various column materials was usually determined using Pasteur pipette columns, as specified in the legends to the figures. In some cases the binding capacities were also determined in test-tubes by mixing samples (1 ml) of the settled gels with solutions (2 ml) containing the protein(s) to be tested (at various concentrations). The mixture was swirled for 10 min at 22°C, the gel was then spun down and the protein concentration in the supernatant was determined. The binding capacities determined by these two methods were in good agreement (within 15%).

RESULTS AND DISCUSSION

The mechanistic studies reported in this paper are based on a comparison of three homologous series of alkyl agaroses (Seph-C_n) whose structures are schematically illustrated in Fig. 2.

(1) The original Seph-C_n series[3,8], denoted Type I, was prepared by the coupling of alkylamines to CNBr-activated agarose. Around neutral pH, the column materials of this series have a net positive charge due to protonation of the substituted

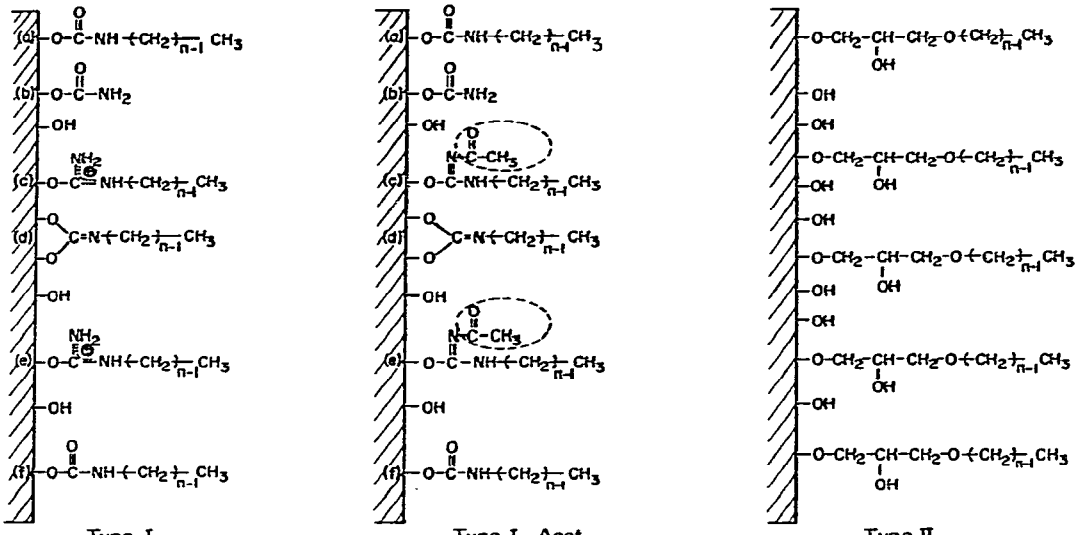


Fig. 2. Schematic representation of the structures of the different types of alkylagarose series used in this comparative study. The Type I series is positively charged at neutral pH, whereas the Type I, Acet and Type II series are devoid of electrical charge. Structures (a) and (f) denote substituted carbamate; (b) a non-substituted carbamate formed in the course of the activation step; (c) and (e) denote the substituted isourea linkages which are protonated around neutral pH and thus positively charged; (d) a substituted imidocarbonate; "*n*" denotes the number of carbon atoms in each hydrocarbon chain.

isourea linkages (c and e Fig. 2, left panel) at the "root" of some of the alkyl chains in each of the columns in the series. These cationic groups were shown to have an identical pK_a (9.7)[9–12] in all of the Seph-C_n columns tested (n = 1–12).

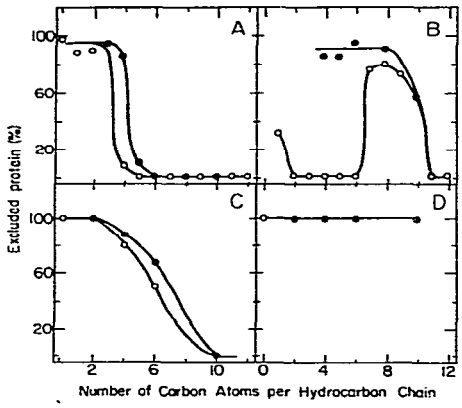
(2) A similar Seph-C_n series derived from the above by acetylation, a procedure which had been shown by Wilchek and Miron[18] to cancel the positive charges of the Type I columns. This modified series, denoted Type I, Acet (Fig. 2, middle panel), was shown to be obtained from the Type I columns without a significant detachment of ligand (less than 10%) and was therefore comparable with its parent column series with respect to its ligand density.

(3) A series of Seph-C_n columns prepared by the coupling of n-alkyl glycidyl ethers[19] to agarose by the procedure described by Hjertén *et al.*[20]. The column materials in this series, denoted Type II (Fig. 2, right panel), are devoid of electrical charge.

The columns used were routinely characterized with respect to their charge density (by potentiometric titrations) and their hydrocarbon chain density (by the use of ^{14}C-labelled ligands[9,26] and/or NMR[21]). It was therefore possible to compare the adsorption properties of column materials which differed in their net charge but were essentially identical in their hydrocarbon-chain densities. This enabled us to assess in a quantitative and meaningful manner the relative contribution of ionic *vs.* hydrophobic interactions to the adsorption properties of the original (Type I) Seph-C_n columns[3,8].

The experiment depicted in Fig. 3 represents a comparison of the adsorption properties of Seph-C_n columns (prepared by the CNBr procedure) before and after

acetylation with acetic anhydride. It thus addresses itself to the question of the extent to which the adsorption of water-soluble proteins to Type I Seph-C_n columns depends on electrostatic interactions with the column materials. It is evident from Fig. 3 that in the cases of glycogen phosphorylase *b*, human hemoglobin and lysozyme, acetylation of the columns does not significantly alter the adsorption profile (or lack of adsorption) of the proteins. A similar result (not illustrated) was also obtained with bovine serum albumin. These results would suggest that at least in these cases, the net positive charge of the column materials is not essential for adsorption, and in fact does not contribute much to it.



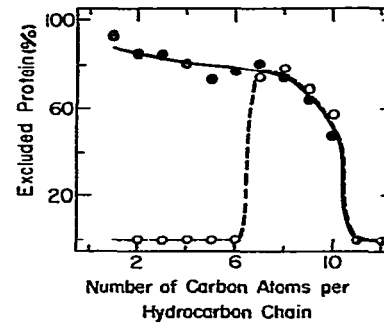


Fig. 3. Adsorption profiles of different proteins on a series of Seph-C_n columns before (○; Type I) and after (●; Type I, Acet) acetylation of the column materials with acetic anhydride. Samples (1 mg) of the following proteins were applied, each on a column (5 × 0.5 cm I.D.) equilibrated and run at 22°C with the indicated buffer: (A) glycogen phosphorylase *b* (in 50 m*M* sodium β-glycerophosphate, pH 7); (B) ovalbumin (in 50 m*M* Tris · HCl, pH 8); (C) hemoglobin (in 0.1 *M* sodium phosphate, pH 7); (D) lysozyme (in 0.1 *M* sodium phosphate, pH 7). The first 12 ml emerging from the columns were collected and their absorbance at 280 nm was measured and used to calculate the percentage of excluded protein in each case.

Fig. 4. Effect of the buffer composition on the adsorption profile of ovalbumin on Seph-C_n (Type I) columns. Samples (1 mg) of ovalbumin were applied on a homologous series of the Type I columns equilibrated and run with: (A) ○, 50 m*M* Tris · HCl, pH 8; (B) ●, 100 m*M* sodium phosphate, pH 7. All other experimental conditions were identical with those in the experiment depicted in Fig. 3B.

In the case of ovalbumin, however, there seemed to be a dramatic difference in the adsorption profile depending on whether the Seph-C_n columns were acetylated (●, Fig. 3B) or not (○, Fig. 3B). This difference was very pronounced for the columns with shorter hydrocarbon chains ($n \leqslant 6$). For example, in the cases of $n = 4$, 5 and 6, ovalbumin was retained on the Type I (charged) columns but was excluded from the Type I, Acet (electrically neutral) columns. This fact is in agreement with the observation of Wilchek and Miron[18] that whereas Type I Seph-C_4 and Seph-C_6 columns adsorb ovalbumin in a 50 m*M* Tris · HCl buffer, pH 8.0, the corresponding acetylated columns fail to do so under the same experimental conditions. However, because the adsorption properties of ovalbumin were studied here with the whole homologous series and not with two columns only, it was immediately realized that the adsorption profile of ovalbumin under these experimental conditions is very unusual, not only when compared with the other profiles depicted in Fig. 3 but also

when compared with the adsorption profiles of a large number of other proteins previously tested under a variety of conditions, where it has been shown[2,3,8–11] that upon increasing the hydrocarbon chain length, the ability of the Seph-C_n columns to adsorb a given protein passes from no retention (total exclusion) through retardation, to reversible binding, up to very tight binding (practically no exclusion).

In view of the fact that we had previously shown[3,4,8–11,27,28] that the ionic composition of the medium can sometimes be a decisive factor in bringing about adsorption or elution of a protein during hydrophobic chromatography or affinity chromatography, we considered the possibility that in the case of ovalbumin the abnormal adsorption profile observed in 50 m*M* Tris · HCl (pH 8.0) might be associated with buffer-dependent specific ion effects[3,4,27,28]. We therefore tried to find out whether by changing the ionic composition of the medium we could get ovalbumin to display a "normal" pattern of adsorption (increased adsorption with increasing hydrocarbon-chain-length). As seen in Fig. 4, this was found to be the case: when the Tris · HCl buffer mentioned above is replaced by 0.1 *M* sodium phosphate, pH 7.0, ovalbumin not only exhibits a "normal" adsorption profile on the Type I columns, but this profile is essentially identical with that obtained with the Type I, Acet columns. It seems therefore reasonable tc assume that the abnormal behaviour of ovalbumin in the Tris · HCl buffer reflects a unique (buffer-dependent) structural change in the protein itself, which alters for example the size, number of lipophilicity of its available hydrophobic patches. Such buffer-dependent structural changes (or "deformation") which affect specific sites in a protein have been not only documented but put to use in structure–function studies[29,30].

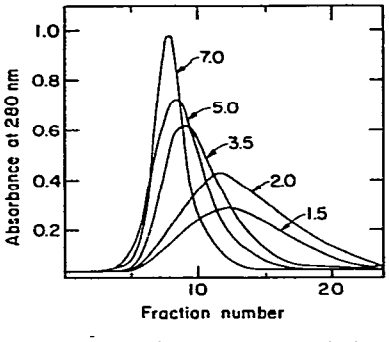
These findings emphasize the need to explore homologous series of columns[2,3] (rather than select one or two randomly chosen members) and to consider a possible interference of specific ion effects[3,8,27,28] before drawing general mechanistic conclusions from the anomalous behaviour of some proteins on some columns under a specific set of conditions.

The lack of adsorption of lysozyme on both the non-acetylated and the acetylated columns is of special interest. Hofstee[5] has proposed that the binding of proteins on to Type I columns depends on a co-operation between hydrophobic and electrostatic forces. He further suggested[5] that the overall net charge of a protein determines whether it will bind to such columns or not. This suggestion was based on the observation that basic proteins such as lysozyme, which are positively charged at neutral pH do not bind to Seph-C_1, Seph-C_4 or Seph-C_8 in the Type I series at pH 8.0 and low (0.05) ionic strength. In principle, this lack of binding may be due indeed to an electrostatic repulsion between the positively charged columns of the Type I series and the positively charged protein in question, but it might also be due to the fact that under the conditions chosen for the experiment such proteins may simply not possess available hydrophobic patches on their surface for hydrophobic interaction with the column materials.

Mohammadzadeh *et al.*[31] compared the binding of *n*-heptane to various proteins, and showed that in contrast to proteins like bovine serum albumin, β-lactoglobulin and turkey ovomucoid, lysozyme has an exceptionally low capacity for binding *n*-heptane at neutral pH. However, on lowering the pH of the solution to 2.0 there is a dramatic increase (*ca.* nine-fold) in the binding of *n*-heptane to lysozyme. This increased binding was attributed to a conformational change in lysozyme, as a result of

which hydrophobic binding sites become accessible to interaction with *n*-heptane[31]. In view of the fact that on lowering the pH of the medium the net positive charge of both the protein and the Type I Seph-C_n columns could only increase, one would except the electrostatic repulsion between them to at least stay the same if not increase, so that at low pH no adsorption should occur, if indeed these columns function by virtue of their positive charges. On the other hand, if the retention power of the columns arises from hydrophobic interactions, then lowering the pH might result in an adsorption of lysozyme by the columns, just as the lowered pH promotes the capacity of this protein to bind *n*-heptane.

Indeed, when lysozyme is applied on an exploratory kit of the Type I columns equilibrated and run at various pH values, less and less protein is excluded from the higher members in the series ($n \geqslant 5$) as the pH of the eluent is lowered to 1.5–2.0[9,10]. This gradual increased retardation with decreasing pH can further be seen in the retardation profile of lysozyme on Type I Seph-C_{10} (Fig. 5). The fact that the binding or lack of binding of lysozyme to the columns does not depend on their net charge is further illustrated in the experiment depicted in Fig. 6, which clearly shows that the elution profile of lysozyme on Seph-C_{10} is identical on the acetylated and non-acetylated columns both when the columns exclude the enzyme (at pH 7) and when they retard it (at pH 2).



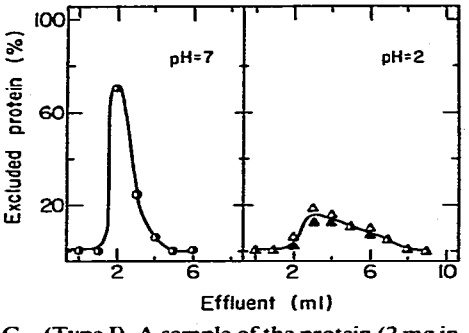


Fig. 5. Effect of pH on the retardation of lysozyme on Seph-C_{10} (Type I). A sample of the protein (2 mg in 0.2 ml) was applied on each of five identical columns (15 × 6 cm I.D.) which were equilibrated and run at 22°C with the following buffers: 0.1 *M* sodium citrate–phosphate, pH 7.0, 5.0 or 3.5; and 0.1 *M* KCl–HCl, pH 2.0 or 1.5. Fractions of 1.2 ml were collected and their absorbance at 280 nm was monitored.

Fig. 6. The net charge of a Seph-C_{10} column has no effect on the retardation of lysozyme by the column. Samples of the protein (1 mg) were applied on either Type I (○, △) or Type I, Acet (●, ▲) columns (5 × 0.5 cm I.D.), equilibrated and run (22°C) with either 0.1 *M* sodium phosphate buffer (pH 7, left panel) or 0.1 *M* KCl–HCl buffer (pH 2, right panel). Fractions of 1 ml were collected and their absorbance at 280 nm was used to calculate the percentage of excluded protein in each fraction.

It could thus be concluded that the lack of adsorption of lysozyme on to the charged (Type I) columns is due to the fact that in its native conformation lysozyme lacks accessible hydrophobic patches. Under conditions where lysozyme assumes a conformation in which hydrophobic regions become accessible (as indicated by a capacity to bind non-charged free heptane molecules) then this protein is retarded by the Type I Seph-C_{10} column. As expected, under these conditions the retardation occurs whether this column is charged (Type I) or not (Type I, Acet).

Additional sets of comparable alkylagaroses with and without charge were obtained by using the CNBr coupling method[3,8] on the one hand, and the glycidyl ether coupling method on the other (the latter coupling method having been introduced for hydrophobic interaction chromatography by Hjertén and co-workers[20]). One such set consisted of two homologous series of Seph-C_n columns (Type I and Type II) in which all the individual members had a hydrocarbon chain density of 10 $\pm$ 1 μmole per gram of gel. Three proteins (bovine serum albumin, myoglobin and β-lactoglobulin) were applied on each column of these two homologous series. As seen in Fig. 7, the two types of column (charged and non-charged) exhibited very similar (if not identical) adsorption profiles for each one of the proteins.

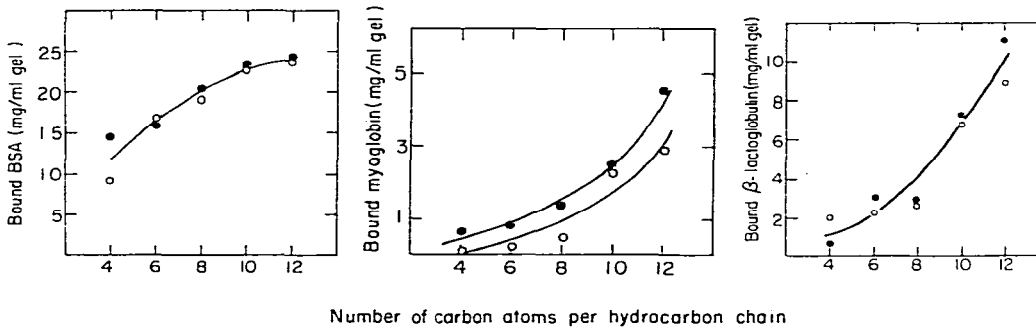


Fig. 7. Similarity in the adsorption profiles of three proteins on two homologous series of Seph-C_n columns: one series positively charged (●, Type I), and the other series electrically neutral (○, Type II). The column materials used in this experiment were very similar with respect to their ligand density (in all cases 10 $\pm$ 1 μmole of ligand per gram of gel). Left panel: Samples of bovine serum albumin (25 mg in 0.5 ml of a buffer composed of 10 m*M* sodium phosphate and 90 m*M* NaCl, pH 7) were applied on each of the Seph-C_n columns (*ca.* 4 × 0.6 cm I.D., gel volume 1 ml) which were equilibrated and run (22°C) with the same buffer. The protein bound to a given column was calculated by subtracting the amount of protein excluded in the first 2 ml of emerging fractions from the total amount of protein applied on the column. Central panel: Samples of myoglobin (6 mg in 0.15 ml of a buffer similar to the above, but at pH 5.3) were applied and run on sets of Seph-C_n columns as above. Right panel: Samples of β-lactoglobulin (20 mg in 0.4 ml of the buffer described in the legend to the left panel in this figure) were used. All other experimental details as described above.

The effect of ionic strength and of ligand density on the adsorption capacity of Seph-C_{12} (Type I) and Seph-C_{12} (Type II) is shown in Fig. 8. It can clearly be seen that while there is some difference in the binding curve of the protein tested (myoglobin) to these two types of columns, when run at a very low ionic strength (0.01 *M* phosphate buffer), this small difference becomes negligible when the ionic strength of the medium is increased to the physiological range (by addition of NaCl to a final concentration of 0.09 *M*), which has been routinely used in hydrophobic chromatography[3,4,8–13]. It is interesting to note that on increasing the concentration of NaCl *ca.* 40-fold (from 0.09 *M* to 3.99 *M*) there is a significant increase in the adsorption of myoglobin on to Seph-C_{12}. However, this increase occurred with both the Type I and Type II columns, and the binding curves of myoglobin on Seph-C_{12} columns with increasing ligand densities were practically identical for the positively-charged and the neutral columns (Fig. 8).

In an attempt to clarify whether the experiments described above represent exceptional cases rather than the general rule, we applied a crude muscle extract

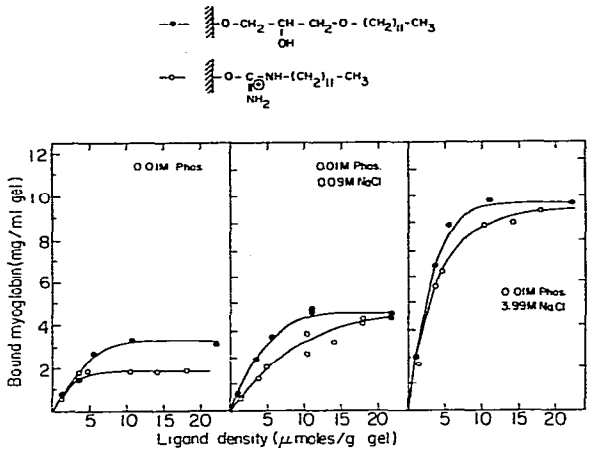


Fig. 8. Saturation curves of myoglobin on charged (●, Type I) and electrically neutral (○, Type II) Seph-C_{12} columns with various ligand densities, and the influence of ionic strength on these curves. Samples of myoglobin (10 mg in 0.25 ml of a buffer composed of 10 m*M* sodium phosphate and the indicated concentration of NaCl, pH 5.3) were applied on each of the Seph-C_n columns (*ca.* 4 × 0.6 cm I.D., gel volume 1 ml) which were equilibrated and run (22°C) with the same buffer. The protein bound to a given column was calculated by subtracting the amount of protein excluded in the first 2 ml of emerging fractions from the total amount of protein applied on the column.

containing a variety of proteins in different concentrations on two homologous series of columns (Type I and Type II), which were comparable with respect to their ligand density. The protein mixture excluded from the columns was then subjected to polyacrylamide gradient gel electrophoresis in the presence of sodium dodecylsulphate. As evident from Fig. 9, on increasing the hydrocarbon chain length, less and less proteins are excluded, indicating that more and more proteins become adsorbed on to the columns. However, on comparing pairs of columns with the same hydrocarbon chain length (Type I *vs.* Type II) it can readily be seen that there is a remarkable similarity in the composition of the fractions excluded from the charged or non-charged columns. In fact, with the exception of very minor differences (see for example the case of Seph-C_{10}), the two parallel columns seem to retain in each case the same set of proteins and to the same extent (Fig. 9).

When the composition of the excluded protein mixtures of the various columns was determined quantitatively by densitometry of the gels, the similarity between the binding properties of any Type I column and its companion Type II column was even more apparent (*cf.* the densitometric tracings of the gels obtained from Type I and Type II Seph-C_4 and Seph-C_{12} columns, Fig. 10). Furthermore, by measuring the area of each individual peak (denoted A to O) in tracings obtained from all the gels depicted in Fig. 9, it could be shown that the retention power of the Type I and Type II columns for each one of the fifteen distinct proteins in the mixture used was essentially identical, because the differences observed (filled *vs.* empty circles in Fig. 11) were not greater than those observed with two different batches of either type of Seph-C_n column.

It was originally stressed[3] that the distribution of hydrocarbon chains affects the binding of proteins on the Seph-C_n columns, because as the density of the hydro-

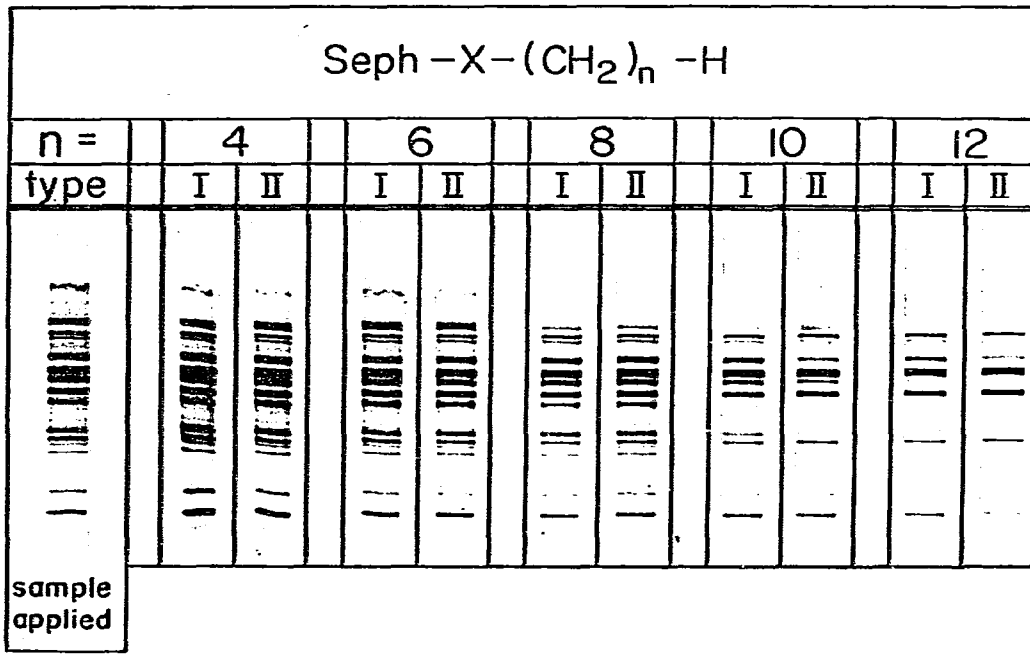


Fig. 9. A comparison of the adsorption properties of charged Seph-C_n columns (Type I) with those of electrically neutral Seph-C_n columns (Type II). The columns compared here had identical hydrocarbon chains (equal number of carbon atoms per hydrocarbon chain, n) and very similar ligand densities (in all cases 10 ± 1 μmole per gram of gel). Samples (0.4 ml containing 4–5 mg protein) of crude muscle extract (dissolved in a buffer composed of 10 m*M* sodium phosphate and 90 m*M* NaCl, pH 7), were applied each on a column (*ca.* 4 × 0.6 cm I.D., gel volume 1 ml), equilibrated and run (22°C) with the same buffer. The excluded protein (first 2 ml) was collected and an aliquot (10 μl) of this fraction was subjected to electrophoresis on a polyacrylamide gradient gel, 7–20% in the presence of sodium dodecylsulphate. Note the gradual disappearance of some of the proteins (*i.e.* their gradual adsorption on to the columns) with increasing hydrocarbon chain length (n), and the remarkable similarity in the adsorption properties of the charged (Type I) and the electrically neutral (Type II) column materials having an equal value of n.

carbon ligand increases, such a column can offer more hydrocarbon chains for interaction with the hydrophobic patches of any given protein and therefore its tightness of binding would often increase[9]. Thus, proteins that under a given set of conditions would be excluded or slightly retarded on a column with a low ligand density may become retained when this density is increased, a phenomenon which has been extensively studied by Jennisen and Heilmeyer[32,33].

As seen in Fig. 12, on increasing the ligand density of Seph-C_{12} columns *ca.* ten-fold (from *ca.* 1 μmole of ligand per gram of gel to *ca.* 11 μmole/g) there is a considerable change (under the same experimental conditions) in the population of proteins that are excluded from the columns, and consequently in the population of proteins that become adsorbed on to the columns. It is therefore obvious that it would be meaningless to compare charged *vs.* non-charged columns unless they are previously shown to have very similar, preferably identical, ligand densities. In the absence of such similarity, no conclusion can be drawn regarding the contribution of charge to the adsorption properties and to the resolution power of Seph-C_n columns. On the other hand, when charged and non-charged column materials are compared,

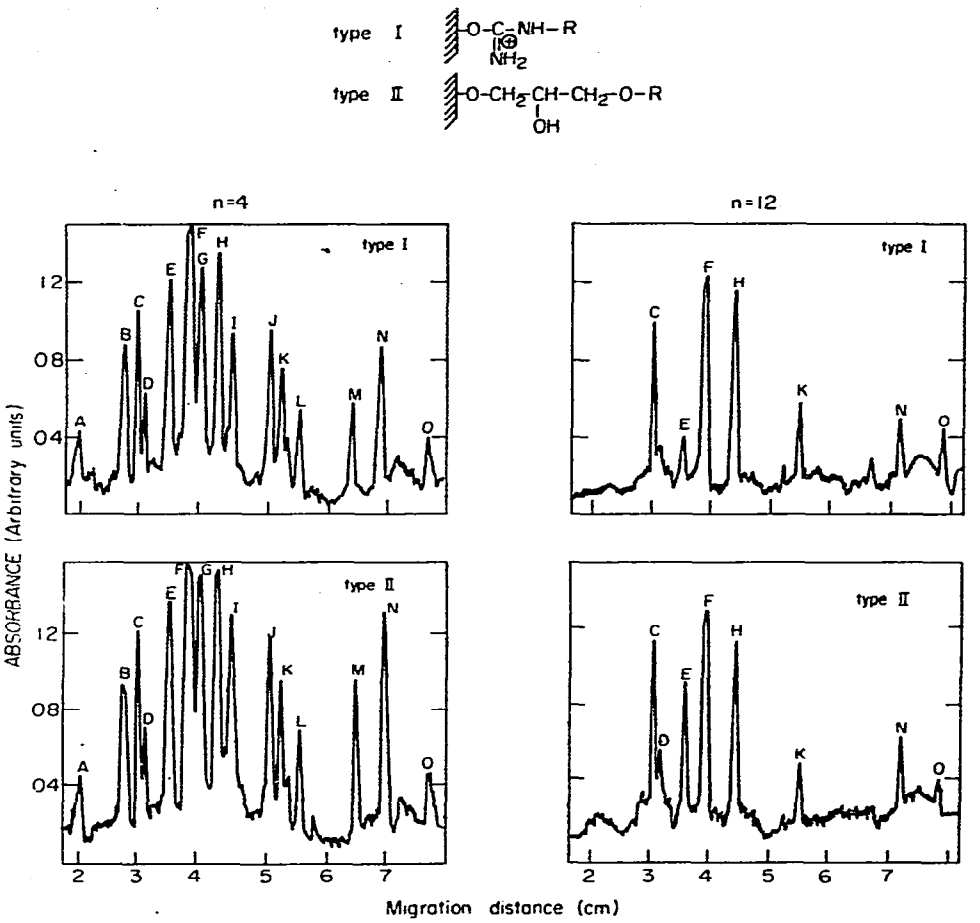


Fig. 10. Densitometric scans of some of the gels depicted in Fig. 9 for a quantitative assessment of the adsorption properties of Type I *vs.* Type II columns ($n = 4$ and $n = 12$).

each with its companion column of comparable ligand density (Fig. 12 and 13) it becomes evident that the contribution of the charges* (in the Type I columns) to the adsorption properties of the columns is practically negligible and that "hydrophobic interactions must contribute dominantly to the retention and discrimination power of these columns"[4].

In view of the fact that with the original Type I columns, it is often possible to elute some proteins from the columns by raising the ionic strength of the irrigating

* It should be noted that in a large number of column materials characterized in our laboratory[26,34] with respect to their charge density and hydrocarbon chain density, it has been consistently observed that the charge density ranges between 50 and 70% of the ligand density (*cf.* Fig. 12, right panel), suggesting that not every hydrocarbon chain is anchored to the matrix backbone through a substituted isourea linkage. This would indicate the occurrence of some heterogeneity in the functional groups linking the ligand to the matrix, as originally proposed by Axén and Ernback[14] and by Ahrgren *et al.*[15] (*cf.* also Fig. 2).

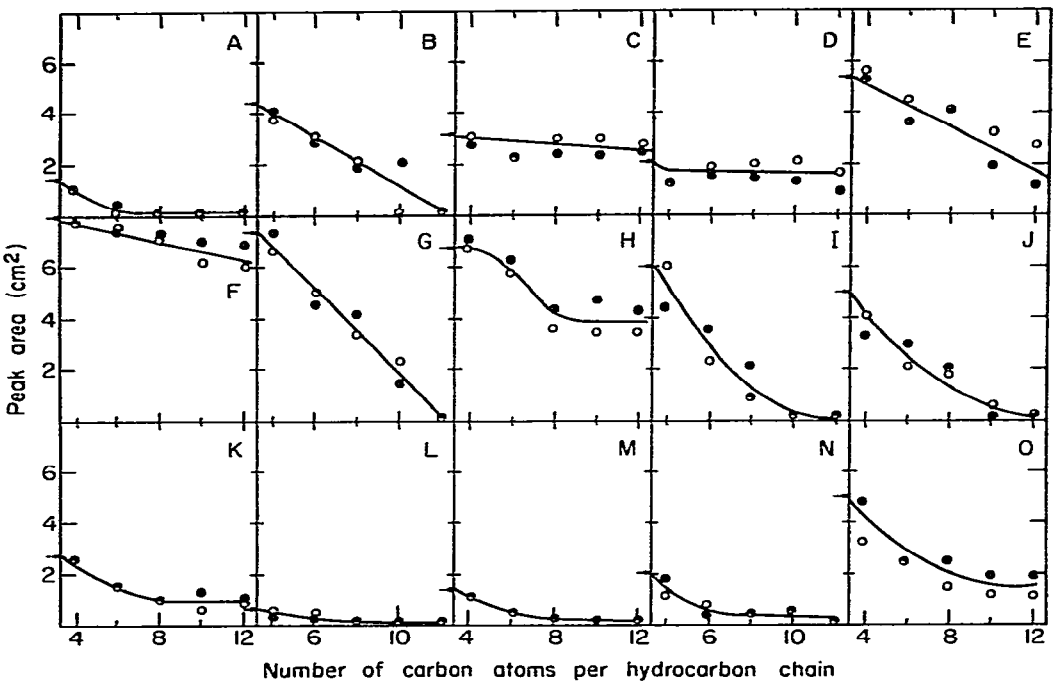


Fig. 11. Exclusion profiles of each of the individual proteins (A to O) in the protein mixture of the crude muscle extract used in the experiment depicted in Fig. 9. These profiles were obtained from densitometric scans of each of the gels shown in Fig. 9 by measuring the relative area of the peaks A to O (cutting out the peaks, weighing them and dividing these weights by that of 1-cm² areas of the same paper). ●, Type I columns; ○, Type II columns.

buffer, some investigators[5,17,18] argued that ionic interactions play a major role in the function of these columns. The quantitative, comparative study presented here, together with the substantial body of evidence reported previously[8–13], would clearly favour our original proposal[2,3] regarding the mechanism of action of Seph-C_n columns.

Generally speaking, the conditions required for elution of a molecule from a column can give an indication as to the type of interaction involved in its retention by this column. This is simple and clear-cut when dealing with a rigid molecule, where it is justifiable to assume that the structure of the molecule is not affected by the change in conditions introduced for the purpose of elution. However, when dealing with proteins that do not have a rigid structure and that are known to undergo conformational changes readily as a result of a change in their environment (ionic strength, ionic composition of the buffer, pH, ambient temperature, presence of deforming agents, surfactants or polarity-reducing agents), the above-mentioned assumption cannot be made and therefore the mechanism of adsorption cannot be deduced from the conditions required for elution. It is well established now that the native conformation of proteins is maintained by and therefore dependent on a large number of intramolecular interactions (ionic, hydrophobic, hydrogen-bond, etc.). Therefore, on changing the environment of a protein molecule it is not possible to avoid a perturba-

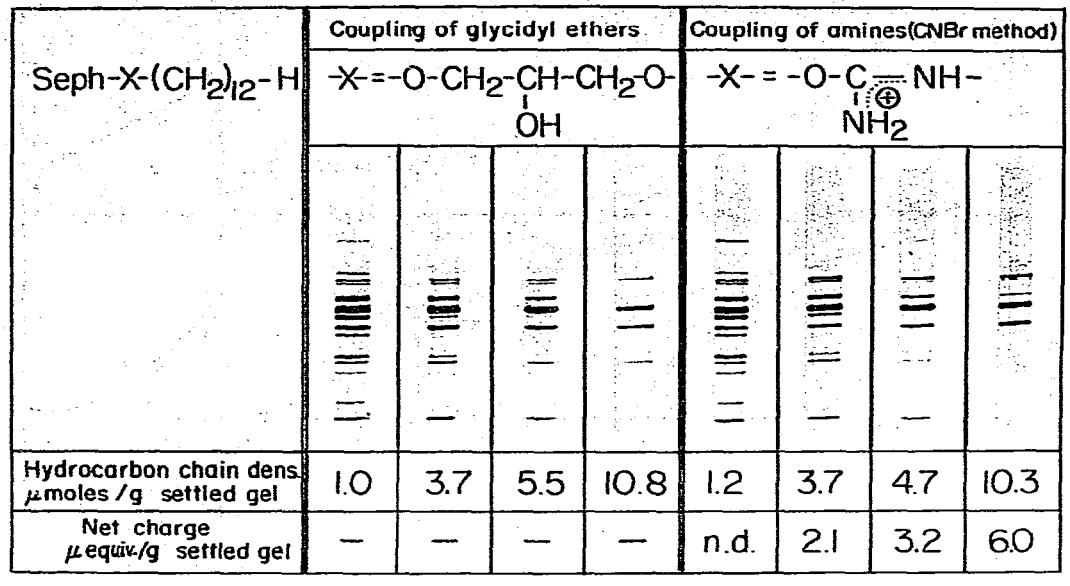


Fig. 12. A comparison of the adsorption properties of Type I (charged) and Type II (electrically neutral) Seph-C_{12} columns varying in their hydrocarbon chain densities. Samples (0.4 ml) of crude muscle extract (dissolved in a buffer composed of 10 m*M* sodium phosphate and 90 m*M* NaCl, pH 7) were applied each on a column (*ca.* 4 × 0.6 cm I.D., gel volume 1 ml), equilibrated and run (22°C) with the same buffer. The excluded protein (first 2 ml) was collected and an aliquot (10 μl) of this fraction was subjected to electrophoresis on a polyacrylamide gradient gel, 7–20% in the presence of sodium dodecyl sulphate. Note the gradual disappearance of some of the proteins (*i.e.* their gradual adsorption on to the columns) with increasing hydrocarbon chain density, and also the remarkable similarity in the adsorption properties of the charged (Type I) and the electrically neutral (Type II) Seph-C_{12} columns with comparable ligand densities.

tion of these interactions and a subsequent change in its conformation. In fact, there are numerous reports in the literature describing dramatic conformational changes caused by moderate modulations of the above-mentioned parameters. To cite just one example: the free catalytic subunit of rabbit muscle cAMP-dependent protein kinase undergoes a pronounced conformational change (detected by a 4.5-fold *increase* in the reactivity of one sulphydryl and a concomitant 3.8-fold *decrease* in the reactivity of another sulphydryl in the enzyme) when the ionic strength is increased from 0.03 *M* to only 0.22 *M*[35,36].

Thus, the effect observed on changing the eluent will be the net result of the influence of this change on: (1) the conformation of the protein (possibly also of the column material); (2) the interactions between the hydrocarbon chains of the column and the hydrophobic sites on the protein surface. Therefore, an increase in ionic strength may sometimes promote retention, sometimes promote elution or may have no effect at all.

It is shown above that structurally comparable Seph-C_n columns, charged and non-charged, display a remarkable qualitative and quantitative similarity in their adsorption and resolution properties. Furthermore, when an increase in ionic strength enhances the binding of a protein to a Seph-C_n column, the enhancement occurs whether the column is charged (Type I) or neutral (Type II) and it occurs to

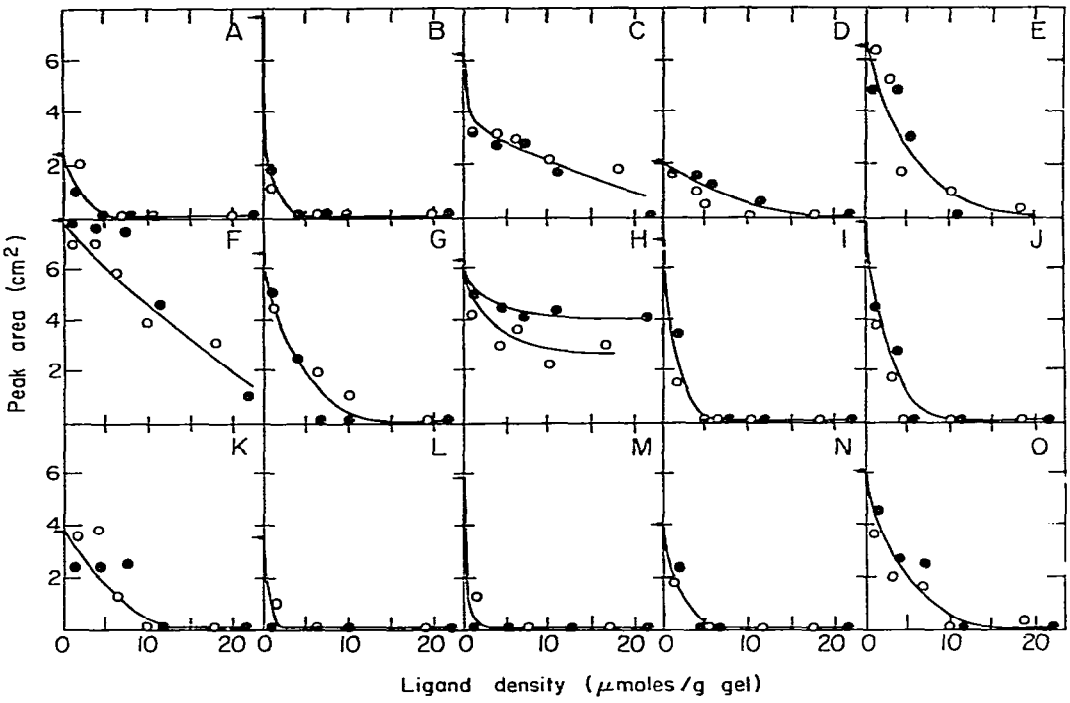


Fig. 13. Exclusion profiles of each of the individual proteins (A to O) in the crude muscle extract used (see Fig. 9, left panel) on Type I (●) and Type II (○) Seph-C_{12} columns with various ligand densities. These profiles were obtained from densitometric scans (similar to those shown in Fig. 10) of the gels depicted in Fig. 12 and additional gels. The areas of the peaks A to O were measured by cutting them out, weighing them and dividing these weights by that of 1-cm^2 areas of the same paper.

the same extent (Fig. 8). Similarly, altering the pH of the irrigating buffer has identical consequences for the retention properties of a Seph-C_n column whether it is charged or not (Fig. 6). These results lead us to conclude that the mechanism of action of Seph-C_n columns involves mainly hydrophobic interactions between the hydrocarbon chains offered by the columns and accessible hydrophobic patches on the protein surface.

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