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PHYSICAL CHARACTERISTICS AND PROPERTIES OF NEW CHROMATOGRAPHIC PACKING MATERIALS FOR THE SEPARATION OF PEPTIDES AND PROTEINS

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SUMMARY

Bonded-phase silica gels were optimized for peptide and protein separations. The optimization involved choice of alkyl chain length and composition, as well as pore sizes of the silica gels. The identities of the alkyl chains were examined by use of solid-state ^{13}C and ^{29}Si cross-polarization magic angle spinning nuclear magnetic resonance spectroscopy (CP/MAS NMR). Furthermore, ^{13}C CP/MAS NMR spectroscopy was used together with elemental analysis for monitoring the synthesis.

It was shown that the selectivity for peptides was affected by changing the ligand density. Optimal resolution of proteins was obtained with a low substitution of octyldimethylsilyl chains in combination with *ca.* 80% of trimethylsilyl chains. The influence of column length and flow-rates on the separation of proteins of similar hydrophobicity, as well as an on/off mechanism in short columns for proteins with a large difference in hydrophobicity, is discussed.

INTRODUCTION

The introduction of bonded-phase silica for reversed-phase chromatography (RPC) of small organic molecules has met with enormous success, because the resolution is high and handling is easy. In the late 1970s and the early 1980s attempts were made to extend RPC to the separation of polypeptides and proteins in the same columns as those used for the separation of smaller molecules.

Two main types of alkylsilane are used in the synthesis of chromatographic packing materials for the separation of small molecules: monofunctional and trifunctional reagents which, when partially hydrolysed, create silanol groups. These may react further, either with the solid phase or by polymerization in solution. Such reactions can be avoided by rigorous control of the reaction conditions, but may also sometimes be deliberately encouraged because they result in an increased hydrophobicity of the gels.

Most commercially available RPC gels consist of silica with pore diameters of 50–100 Å, substituted with octadecyl alkyl chains. Ordinarily, the alkylating agent is used in excess in order to cover as many silanol groups as possible. However, for steric reasons large numbers of residual or active silanols are left after alkylation.

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Those groups may be covered by a second reaction with chlorotrimethylsilane, the endcapping reaction, when these residual groups are thought to react[1]. However, it has been shown by using the endcapping reagent alone that only about half of the silanol groups on the silica surface are available for reaction[2]. There are two reasons for this incomplete reaction: (1) a bulky trimethylsilyl group will prevent reaction with neighbouring silanol groups during normal reaction conditions, and (2) silica gels contain micropores where the silanol groups are effectively shielded. The situation is, of course, even worse in the case of the bulky octadecyl silane.

The effect of endcapping has been discussed since the first bonded-phase silica was introduced. This discussion has concentrated on the influence of silanol groups on the separation mechanism, *i.e.* hydrophobic interaction *versus* mixed-mode separation[3–5].

Most of the chromatographic packings intended for the separation of small molecules are also useable for separating macromolecules of biological origin. However, there are significant differences between packings made for the separation of small molecules and packings optimised for protein separation. The importance of pore size[6,7] and type of silica[8] in protein separation has been discussed.

The mechanisms of reversed-phase separation have also been discussed. The multisite attachment[9] and surface tension[10] alternatives have been examined, and it seems obvious that proteins and polypeptides separate according to a type of mechanism different from the one for small molecules.

Characterization of RPC supports normally includes particle size distribution, pore volume, pore size distribution and surface area. The identity of the chains may be determined by pyrolysis gas chromatography[11] in combination with elemental analysis. Recently, ^{13}C and ^{29}Si cross-polarization magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy was introduced as a technique for the characterization of bonded-phase silica[12]. Bayer *et al.*[13] made an extensive study of commercially available supports. Their conclusion was that it is possible not only to determine the various structural elements on the surface but also to differentiate between the mode of preparation, *i.e.* monofunctional or polyfunctional reagents. CP/MAS also shows whether the support is endcapped or not. By ^{29}Si CP/MAS NMR spectroscopy it is possible to distinguish between signals for the siloxane bond and the silanol group, even if the chemical shift difference is small. As expected, it clearly shows the presence of free silanol groups on bonded-phase supports.

EXPERIMENTAL

Instrumental

The ^{13}C CP/MAS NMR spectroscopy was performed on a JEOL FX 200 at 50.10 MHz. A KEL-F rotor was used with a spinning rate between 3.0 and 3.4 kHz. The contact time was found to have an optimum for our purposes at *ca.* 10 msec. Below 4 msec the signal from the methyl carbon of the long alkyl chain vanished. The repetition time was 1 2 sec. The external reference was hexamethylbenzene at 132.3 ppm. The ^{29}Si CP/MAS NMR spectrum was obtained on a Varian XL-200 at 39.75 MHz. A KEL-F rotor was used with a spinning rate of 2.5 kHz. The repetition time was 4.2 sec, and the contact time 5 msec. The reference was the signal from the silanol group at -100 ppm.

The chromatograms were obtained on a Pharmacia FPLC-system, consisting of two P-500 pumps, an injection valve V-7, GP-250 gradient programmer and a UV-1 monitor, fitted with a HR-10 flow cell. For the chromatograms in Figs. 4 and 5 a LDC HPLC-system was used with two Constametric III pumps, a Rheodyne loop injector, a LDC gradient master, a LDC gradient mixer and a Pharmacia UV-1 monitor, fitted with a HR-10 flow-cell.

Columns

Pharmacia PepRPC hr 5/5, ProRPC 5/2 and ProRPC 5/10 prepacked glass columns with adjustable adaptors, 5 cm, 2.5 cm and 10 cm long, respectively, with I.D. 0.5 cm, were used.

For the chromatograms obtained with the LDC system a Waters Assoc. NovaPak steel column (15 cm × 3.9 mm I.D.) an Alltex Ultrasphere IP steel column (15 cm × 4.6 mm I.D.) and PepRPC HR 5/5 were used. The PepRPC HR 5/5 column was connected to the LDC system via two zero-volume unions (Valco HPLC fittings).

Chromatographic conditions are described in the legends for each figure. Chromatography was performed at room temperature.

Chemicals

Acetonitrile was obtained from Fluka and was of HPLC grade. The water used was of Milli-Q quality (Milli-Q systems for reagent grade water). The PTH-amino acids and bovine insulin was purchased from Sigma. Ribonuclease A, bovine serum albumin and ovalbumin were from Pharmacia's gel filtration calibration kit. All other reagents were of high purity. The haemoglobins used were a gift from Dr. J.-O. Jeppsson (Malmö, Sweden).

Trypsin digestion of globin β-chain was performed at an enzyme to substrate ratio of 5:100 (w/w) in 0.1 *M* ammonium hydrogen carbonate for 3 h at 37°C and terminated by boiling for 1 min. Elemental analyses were performed by Mikrokemi, Uppsala. All gels were dried under reduced pressure at 105°C for 2 h before analysis.

The values in Table I were obtained by using the formula[14]

$$N\ (\text{mol/g}) = \frac{P_c}{1200\ n_C - P_C\ (M - 1)}$$

where P_C is the measured carbon percentage, N is the number of moles of organic phase per gram of unmodified silica, n_C is the number of carbon atoms in the bonded silane molecule, and M is the molecular weight of the silane molecule.

PepRPC

PepRPC is based on a 100 Å spherical silica gel sized to 5 μm. Chlorotriethylsilane (TES) has been chosen in combination with chloro octadecyldimethylsilane (ODDMS) rather than the conventional chlorotrimethylsilane because it afforded a slightly increased resolution of large peptides. Furthermore, it should be able to shield residual silanol groups more efficiently. Using the data in Table I, the molar ratio TES:ODDMS was calculated to be 4.7. The PepRPC was thus calculated to contain 17.5% of ODDMS groups and 82.5% of TES groups.

TABLE I

DATA FOR SILANISED SUPPORTS

Substituent(s)	*Carbon content (%)*	*Surface area (m^2/g)*	*Surface coverage*			*Designation*
			mmol/g	*μmol/m^2*	*nm^{-2}*	
TMS	1.2	68	0.3708	5.02	3.03	C_1-300Å
TMS	4.7	406	1.4410	3.55	2.14	C_1-100Å
TES	6.4	300	0.9606	3.20	1.93	C_2-100Å
ODMS	2.5	68	0.2160	3.18	1.91	C_8-300Å
ODDMS	19.0	406	1.0491	2.58	1.56	C_{18}-100Å
TES	6.8	386	1.0258	2.66	1.60	PepRPC
ODDMS	4.9		0.2180	0.56	0.34	C_2/C_{18}-100Å
TMS	1.0	74.7	0.2834	3.79	2.29	ProRPC
ODMS	0.6		0.0504	0.68	0.41	C_1/C_8-300Å

ProRPC

ProRPC is based on 300-Å spheroidal silica gels, 5 μm in size. For separation of proteins, substitution of the packing material with a combination of trimethylsilyl (TMS) and octyldimethylsilyl (ODMS) was found to give optimal separation properties. Fig. 1 shows the ^{13}C CP/MAS spectrum of the gel. The TMS:ODMS ratio was obtained by correlating the integral over the methyl carbons to the rest of the ODMS chain on a gel with and without any TMS. Thus, the contribution of TMS was determined (Table II) by assuming that the methyl groups of TMS and ODMS have the same mobility. From Table I, the TMS:ODMS molar ratio was obtained and found to be 5.6, or 82% of TMS groups and 18% of ODMS groups.

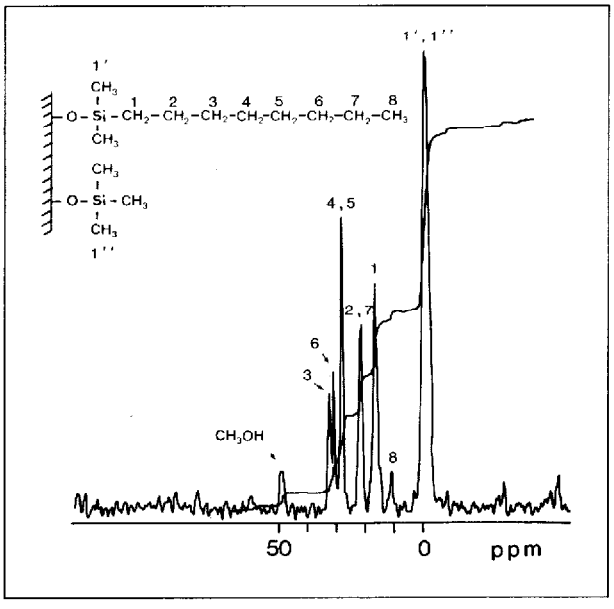

Fig. 1. 50.10 MHz ^{13}C CP/MAS spectrum of ProRPC.

TABLE II

RELATIONSHIP BETWEEN SHORT CHAINS AND LONG CHAINS OF PEPRPC AND PRORPC CALCULATED FROM ELEMENTAL ANALYSIS AND CP/MAS DATA

Support	*CP/MAS TMS: ODMS*	*Ratio TES: ODDMS*	*Total carbon (%)*	*TMS (%)*	*TES (%)*	*ODMS (%)*	*ODDMS (%)*
PepRPC	–	1.4	11.7	–	6.8	–	4.9
ProRPC	1.6	–	1.6	1.0	–	0.6	–

The packing materials in Table I (C_1-300Å, C_1-100Å, C_2-100Å, C_8-300Å and C_{18}-100Å) were prepared according to published methods[14].

RESULTS AND DISCUSSION

New RPC packings have been optimized for peptide and protein separation; for convenience, the names PepRPC and ProRPC, respectively, are used. The preparation involves new syntheses by using a combination of two alkylsilanes of different

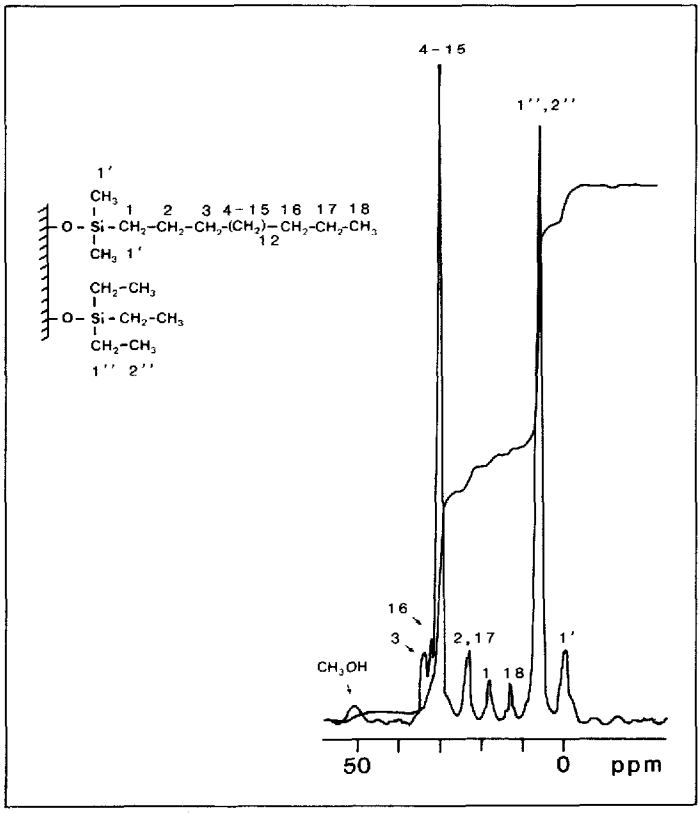

Fig. 2. 50.10 MHz ^{13}C CP/MAS spectrum of PepRPC.

chain length. The ratio of the two chains has been carefully chosen to obtain maximum and uniform substitution of the silanol groups.

In order to monitor the various ratios of triethylsilyl: octadecyldimethylsilyl (TES:ODDMS) during development of PepRPC, solid-state CP/MAS NMR spectroscopy was chosen as the principal tool. Fig. 2 shows the solid-state ^{13}C-NMR spectrum of PepRPC. The carbon atoms of the ODDMS chain have been assigned according to the corresponding ^{13}C NMR spectrum obtained from solution. In TES the two different carbons coincide at 6 ppm. The methyl carbons at *ca.* 0 ppm directly connected to silicon in ODDMS ought to give the same integrated value as the rest of the carbons in the C_{18} chain per carbon atom. As can be seen from Fig. 2, this is not so.

In ^{13}C NMR spectroscopy the intensity of a signal is related to several factors, *e.g.* the spin lattice relaxation time. In solid-state ^{13}C CP/MAS NMR spectroscopy it is the cross-polarization sequence which transfers the magnetisation from hydrogen atoms to carbon atoms. Thus, it can be seen that at a certain contact time (the time that allows this transfer to occur) the integral is quantitative[15]. When the carbons have similar substitution and mobility, the probability of obtaining a quantitative integral increases. In PepRPC, the similarity between the TES group and the two methyl groups in ODDMS was utilized for integration. Because the total percentage of carbon is known, and the integral is assumed quantitative, the contribution from each chain can be calculated (Table II). Those values correlate well with the carbon content obtained in control experiments, where the synthesis was interrupted and the percentage of carbon determined. However, carbon content alone might be misleading, because some results suggest that an exchange reaction may occur during synthesis.

One of the most discussed properties of RPC packings is the presence of residual silanol groups. From Table I it can be seen that by using a combination of short and long chains it is possible to obtain a greater coverage of the surface. PepRPC has a total coverage of 3.22 μmol/m^2 whereas C_{18}-100Å covers only 2.58

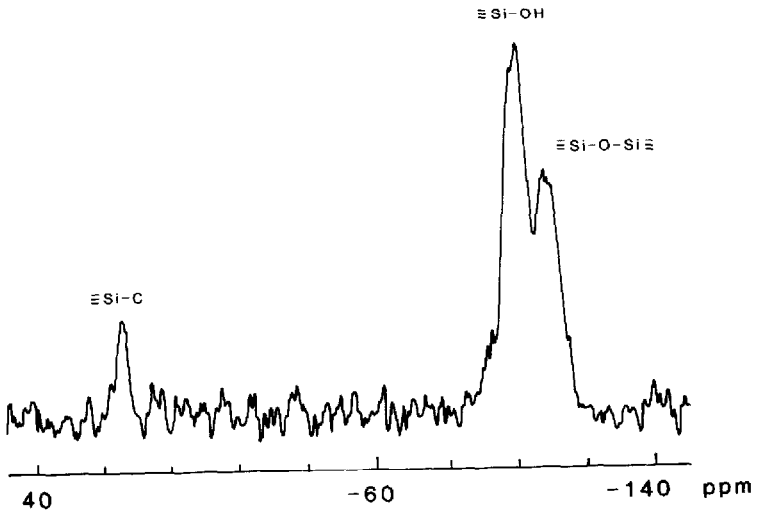

Fig. 3. 39.75 MHz ^{29}Si CP/MAS spectrum of ProRPC.

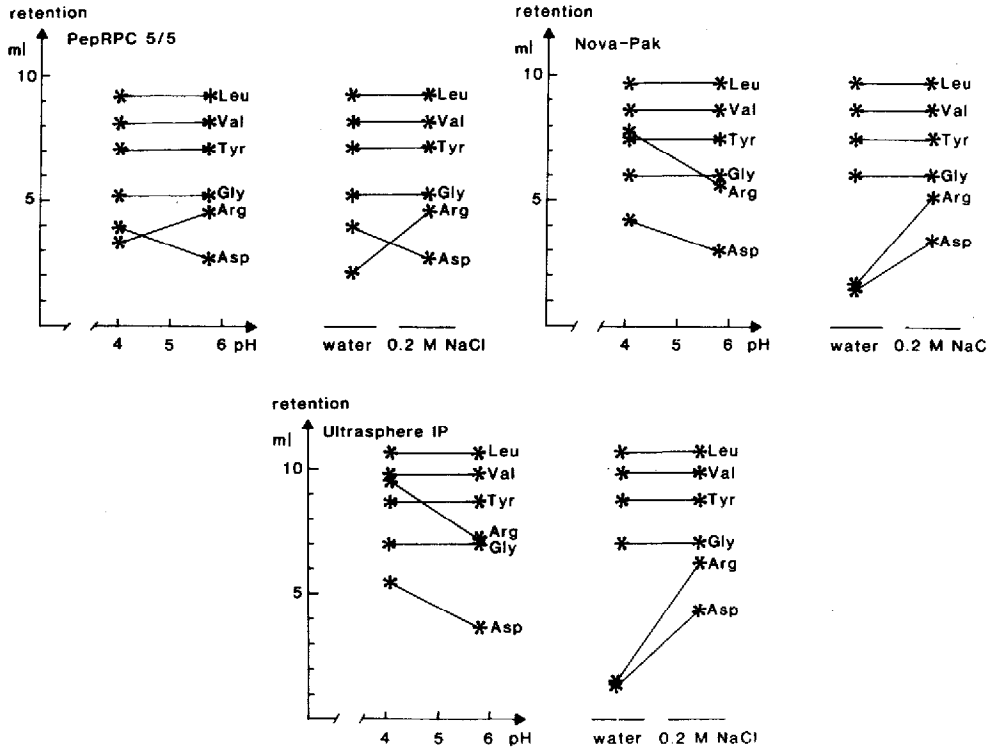

Fig. 4. The retentions of a mixture of six PTH-amino acids on three different columns. Each amino acid (10 mg) was dissolved in 10% of methanol. Injected volume, 20 μl. Gradients: (A) 0.02 *M* potassium acetate pH 4, 5.7, water and 0.2 *M* sodium chloride; (B) acetonitrile 10–70% in 10 min at a flow-rate of 1 ml/min. Top left, PepRPC HR 5/5; bottom, Ultrasphere IP (15 cm); top right, Nova-Pak (15 cm).

μmol/m^2. The highest coverage is, as expected, obtained with TMS alone (3.55 μmol/m^2, C_1-100Å). For ProRPC the corresponding figure is 4.47 μmol/m^2, and for C_8-300Å it is 3.18 μmol/m^2.

In the ^{29}Si CP/MAS spectrum of ProRPC (Fig. 3), the signal just below 20 ppm is due to silicon atoms bound to carbon atoms, and the signals at -100 ppm and -110 ppm from silicon atoms of unsubstituted silanol groups and the siloxane bond, respectively.

The influence of the TES:ODDMS ratio of PepRPC on the retention is most clearly shown in the separation of small molecules by isocratic elution. As expected, the retention is decreased compared with C_{18}-100Å.

Fig. 4 shows the separation of a mixture of six PTH-amino acids on PepRPC and two other column materials. In pure water the charged amino acids, arginine and aspartic acid, are retarded on PepRPC but not on the other columns. In 0.2 *M* sodium chloride the "salting-in effect" is seen to affect two of the columns in the same way. In PepRPC, however, arginine and aspartic acid have changed places. The same situation occurs going from pH 4 to pH 5.7. At pH 4 arginine is much less retarded on PepRPC than on the other columns. Fig. 5 shows the chromatograms of the six PTH-amino acids at pH 4 and 5.7. It is difficult, however, to relate this behaviour to the TES:ODDMS ratio of PepRPC.

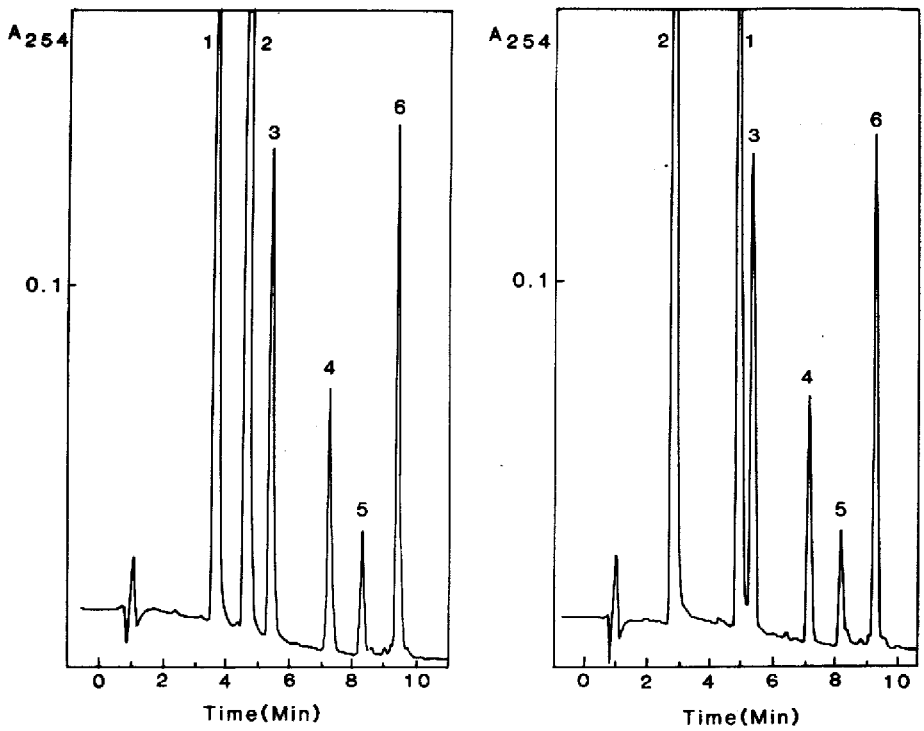

Fig. 5. The elution profiles of a mixture of six PTH-amino acids at two different pH values on PepRPC HR 5/5. Each amino acid (10 mg) was dissolved in 10% of methanol. Injected volume, 20 μl. Gradient: (A) 0.02 *M* potassium acetate; (B) acetonitrile 10–70% in 10 min at a flow-rate of 1 ml/min. Peaks: 1 = arginine; 2 = aspartic acid; 3 = glycine; 4 = tyrosine; 5 = valine; 6 = leucine. Left, pH 4; right, pH 5.7.

A more interesting hypothesis is the formation of clusters of alkyl chains on highly substituted silica gels. The shape of the pores has been shown to change when ODDMS is used in excess[16]. The formation of clusters is discussed by Lochmüller *et al.*[17]. There may be several causes for the presence of clusters. In a dense ODDMS matrix the short distance between the long alkyl chains may by itself create hydrophobic interaction. This is especially relevant when a mobile phase of high water content is used. The forces between the alkyl chains increase owing to the presence of polar solvent, thus forming local hydrophobic sites on the matrix. In syntheses with an excess of ODDMS chloride, the first silane that has reacted may interact with the second and thereby direct the reagent to a neighbouring silanol group, etc. This behaviour depends on the concentration and size of the silane used as well as on the polarity of the solvent. Use of a high-dilution technique and proper choice of solvent should minimize cluster formation. Table I shows that PepRPC has such a low content of ODDMS chains that if substituted randomly on the surface, clusters are improbable. Further investigation on the surface chemistry of silica is in progress.

It was observed during the development of packing materials that the resolution for hydrophobic peptides could be increased by decreasing the amount of ODDMS (PepRPC). This effect might be due to the absence of clusters. Fig. 6 shows

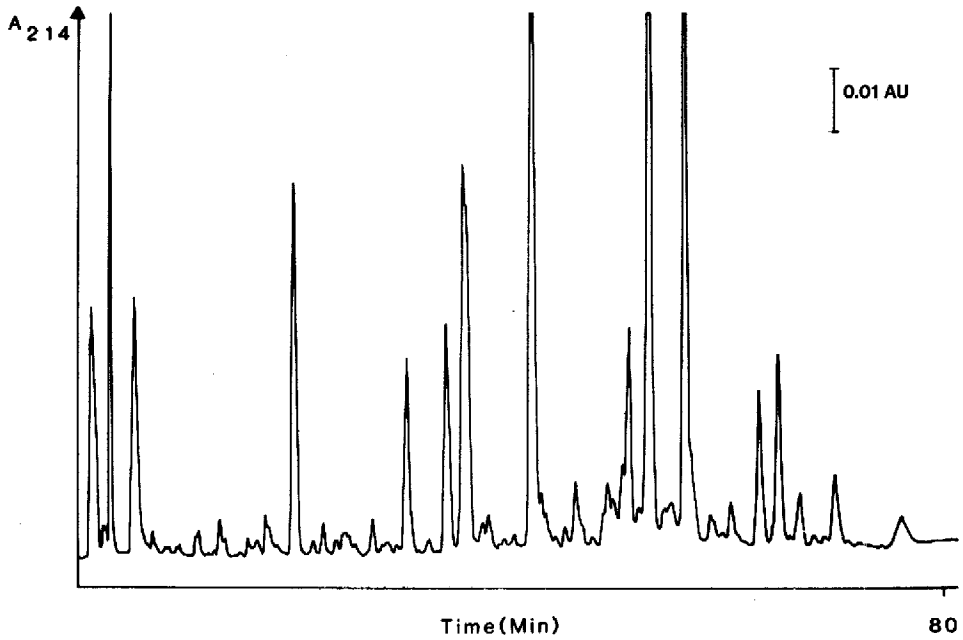

Fig. 6. The elution profile of a tryptic digest of the β-chain of haemoglobin. Conditions: column, PepRPC HR 5/5. Gradient: (A) 49 m*M* dipotassium hydrogen phosphate (pH 2.9); (B) 0–75% acetonitrile in 80 min; flow-rate, 0.5 ml/min.

the chromatogram of a tryptic digest of the β-chain of haemoglobin on PepRPC. The hydrophobic fragments are well resolved without band broadening.

In protein separation there is a trend to use silica gels with larger pore sizes, substituted with shorter alkyl chains. The difference in retention between a 60-Å ODDMS gel compared with a 300-Å ODMS or butyldimethylsilyl gel is small, but the recovery of proteins is significantly higher on a 300-Å gel[7]. It has also been shown that proteins, owing to their strong interaction, are relatively tightly bound to the gel. The proteins are desorbed at about the same concentration of organic solvent regardless of what length of alkyl chain is used ("the protein window"). However, if aromatic substituents are used, the retention can be slightly altered for proteins containing aromatic amino acids. This is probably due to interaction via delocalised π-electrons[6].

In the separation of proteins a suitable modifying ion is normally used as a component part of the separation system. This can be a anion, such as acetate or trifluoroacetate, or an cation, such as trimethylammonium. Two explanations have been given for the influence of this type of modifier on the separation of proteins: (a) the hydrophobic part of the modifier interacts with the alkyl chain, thereby creating an ion-exchanger, and (b) a true ion-pair is formed with the protein, and the separation will then be of reversed-phase type. Both conditions should be sensitive to changes in pH.

The interaction of a protein or a polypeptide with the stationary phase may be described as

$$P_m + nS_{ads} \rightleftharpoons P_{ads} + nS_m \quad (1)$$

where P_m and P_{ads} represent the protein or polypeptide molecules in the mobile phase and the adsorbed state respectively. S_m and S_{ads} represent the corresponding competing mobile phase molecules and n is the number of solvent molecules that must be displaced by the adsorption of the protein or polypeptide molecule.

The retention mechanism of macromolecules in RPC is very complex[18], and eqn. 1 is used to illustrate the behaviour of polypeptides and proteins on ProRPC only in order to explain why it is necessary to have different column lengths. A mechanistic treatment is beyond the scope of this work.

The equilibrium in eqn. 1 is expressed as a static equilibrium. If a flow is introduced through the column, the equilibrium becomes dynamic and it can be shifted by varying the polarity of the solvents in the mobile phase. Because the interaction of proteins and polypeptides in aqueous solution with a hydrophobic stationary phase is strong, the equilibrium in eqn. 1 is shifted far to the right. Application of a steep gradient, with an increasing amount of organic solvent, shifts the equilibrium rapidly to the left. If there is a large difference in hydrophobicity in a mixture of proteins or polypeptides, separation should occur. This may be described as an on/off mechanism. The resolution is then independent of column length and flow-rate.

It can be concluded that for separating a protein or polypeptide mixture with a wide range of hydrophobicities a short column may be used or even preferred. In short columns the recovery should be relatively higher owing to the small amount of gel present, and other column effects should be minimised.

Fig. 7 shows the separation of ribonuclease, insulin, bovine serum albumin and ovalbumin with different column lengths and flow-rates. At 0.2 ml/min, the separation seems to be slightly better on the 2.5-cm column. The on/off mechanism is further illustrated in Fig. 8, where the protein mixture is separated in less than 1 min on a 1.4-cm column.

When the proteins or polypeptides are eluted at a similar composition of organic solvent, it is possible, according to eqn. 1, to let the mobile phase molecules and solute molecules compete for the adsorption sites throughout the whole column. The resolution then becomes dependent on the column length.

The α- and β-chains of haemoglobin were chosen as model compounds because the difference in their interaction with a hydrophobic stationary phase is small. Table III shows the column length dependence on resolution of the α- and β-chains. The formula $R = (V_2 - V_1)/0.5\ (W_1 + W_2)$, where $V_{1,2}$ are the retention volumes of the two chains and $W_{1,2}$ are the bandwidths, has been used to calculate the values in Table III. When the flow-rate and the gradient volume are constant, the resolution increases with increasing column length. However, the resolution also seems to be dependent on the flow-rate, although the data in Table III are inconclusive in this regard.

During this investigation, we also found that retention time and resolution were dependent on the starting conditions. If the concentration of organic solvent in the mobile phase was varied by only a few percent, retention and resolution were affected. This was also noted for larger proteins, such as bovine serum albumin. These results suggest that the tertiary structure of the proteins is altered during chromatography. Further investigation is under way.

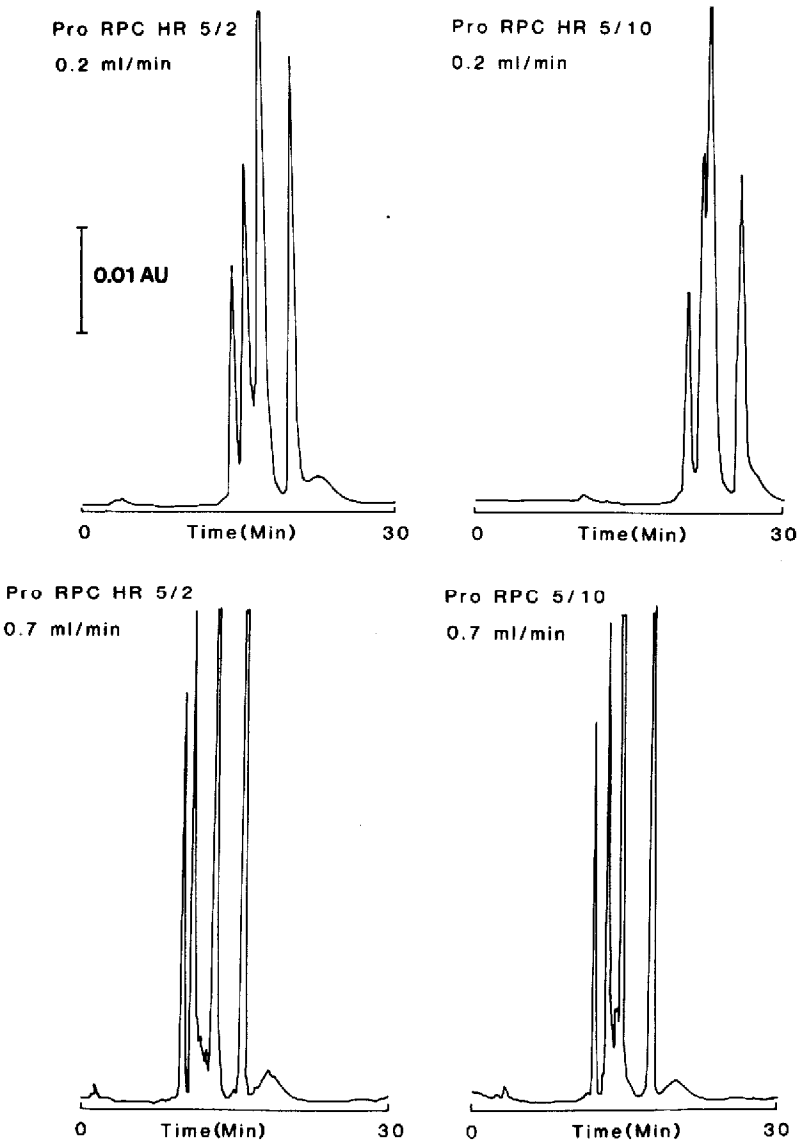

Fig. 7. Influence of column length and flow-rate on the separation of a protein mixture. Gradient: (A) 0.1 trifluoroacetic acid in water; (B) 0.1% trifluoroacetic acid–acetonitrile, 0–100% B in 30 min. Elution order: ribonuclease, insulin, bovine serum albumin, ovalbumin. Top left, ProRPC HR 5/2, flow-rate 0.2 ml/min; top right, ProRPC HR 5/10, flow-rate 0.2 ml/min; bottom left, ProRPC HR 5/2, flow-rate 0.7 ml/min; bottom right, ProRPC HR 5/10, flow-rate 0.7 ml/min. Detector, UV 280 nm.

Using the optimal conditions obtained from Table III, we were able to resolve the globin chains from a heterozygous patient carrying a β-chain mutation (Fig. 9). The primary structures of the two β-chains differ by only one neutral amino acid[19]. This small difference does not account for the the difference in retention. However,

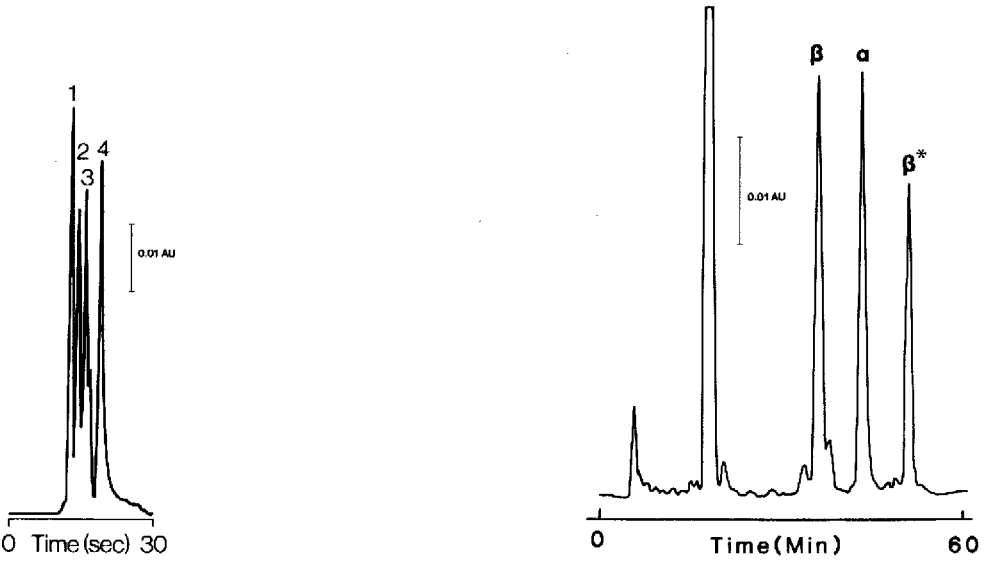

Fig. 8. Fast separation of the same protein mixture as in Fig. 7. Column: ProRPC HR 5/2, packing height adjusted to 14 mm. Gradient: (A) 0.1% trifluoroacetic in water–acetonitrile (9:1); (B) 0.1% trifluoroacetic acid in water–acetonitrile (1:9); 10–75% B in 0.5 min. Flow-rate, 7 ml/min. Elution order same as in Fig. 7. Detector, UV 280 nm.

Fig. 9. Elution profile of globin chains from a heterozygous patient carrying a β-chain mutation. Column: ProRPC HR 5/10. Gradient: (A) 30% trifluoroacetic acid in water–acetonitrile (61:39); (B) 0.15% trifluoroacetic acid in water–acetonitrile (55:45) in 60 min. Flow-rate, 0.2 ml/min. Detector, UV 280 nm.

it is known that mutations in the β-chain might cause drastic changes in the tertiary structure[20,21]. A reasonable conclusion is that, in spite of the denaturing condition caused by 40–45% acetonitrile, there is a conformational change which leads to the large difference in retention seen in Fig. 9.

TABLE III

EFFECT OF FLOW-RATE AND COLUMN LENGTH ON THE RESOLUTION OF α- AND β-CHAINS OF HAEMOGLOBIN (FROM A HAEMOLYSATE)

(A) 39% acetonitrile in 0.3% trifluoroacetic acid; (B) 50% acetonitrile. Linear gradient 0–50% B in 60 min.

Column length (cm)	*Flow-rate (ml/min)*				*Column designation*
	0.2	*0.4*	*0.6*	*0.7*	
2.5	1.4	1.2	0.8	0.9	HR 5/2
5.0	1.9	2.0	1.8	1.4	HR 5/5
7.5	2.3	2.3	2.1	1.9	HR 5/10
10.0	2.9	2.8	2.4	2.4	HR 5/10
10.0	–	–	–	2.1*	HR 5/10

* Linear gradient 0–50% B in 17 min.

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