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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LIII*. EVALUATION OF THE EFFECT OF SEVERAL STATIONARY PHASE PARAMETERS ON THE CHROMATOGRAPHIC SEPARATION OF POLYPEPTIDES ON ALKYLSILICAS

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SUMMARY

The separation of several polypeptides and proteins of varying molecular size by reversed-phase high-performance liquid chromatography on octadecylsilicas of different pore diameters has been studied. In particular, the retention behaviour of phenylalanine oligomers has been further investigated with these stationary phases as the volume fraction, ψ_s, of the organic solvent modifier was systematically increased up to $\psi_s = 0.7$. The results are compared with data obtained in related experiments with commercially available μBondapak C_{18} columns. Quantitative relationships between relative retention, the stationary phase surface area, alkyl ligand density, and ψ_s have been utilised to aid the interpretation of the role of pore size effects in the separtion of these and other polypeptides on alkylsilicas. The results confirm that the s value for a given polypeptide, as evaluated from the linear dependency of the logarithmic capacity factor, log k', on ψ_s and the ω-value as evaluated from the plots of log k' *versus* the surface tension of the eluent, γ, over the range of k' values of interest in isocratic or gradient elution optimisation, are essentially constant for all the octadecylsilicas. Further, the results confirm that retention under regular reversed-phase conditions is directly related to the accessible non-polar surface area within the column. Comparison of the data for small- and large-pore octadecylsilicas suggests that not all of the bonded surface area of packings with small pores (*e.g.*, 7.3 nm) is available to polypeptide solutes. Predictions based on solvophobic theory have been further tested and the linear dependency of the logarithmic selectivity factor (log $\alpha = \tau$) on the bulk surface tension, γ, confirmed. Based on this analysis, the magnitudes of the difference in Van der Waals interaction energies and the free energies of association per phenylalanine residue have been estimated. Finally, the data suggest that the size exclusion component has little effect on reversed-phase retention other than to restrict entropically the access to the inside of the pore. Once inside the pore, retention appears to be predominantly a mobile phase driven phenomenon.

* For Part LII, see ref. 20.

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INTRODUCTION

The separation of peptides and proteins by reversed-phase high-performance liquid chromatography (RP-HPLC) with silica-based stationary phases has attracted much attention during the past few years[1,2]. With complex mixtures of peptides and proteins, adequate control over chromatographic selectivity and solute recovery, in terms of both mass and biological function, are the dominant requirements for successful separations. Numerous studies have favoured gradient elution with acidic, low-ionic-strength mobile phases for either analytical or preparative separations of this class of biomolecules on alkylsilicas. To a very large extent, most investigators interested in the isolation of polypeptides or proteins from biological extracts or in structure determination have, however, relied on empirical selection of a particular set of chromatographic conditions which may be far from optimal in terms of resolution or recovery. Although the practical potential of the technique has found expression in such application studies, detailed understanding of the underlying mechanism of these separations on alkylsilicas or, alternatively, general strategies for optimising resolution still remain poorly developed. In contrast to the separation of low-molecular-weight simple organic acids or bases, the separation of polypeptides and proteins on alkylsilicas is now known to involve generally much more complex distribution equilibria and associated kinetic processes. These complex phenomena arise from a variety of reasons, including multisite interaction with the stationary phase[3–5], from specific solvation equilibria[6], from solute–solute interactions in the mobile phase and at the stationary phase surface[4,7] and solute susceptibility to specific effects mediated by changes in buffer-ion concentration or composition and the extent of ionisation[4,8]. Both of the latter effects can dramatically influence solubility parameter and retention relationships with alkylsilicas. As a consequence of these composite phenomena, tactics for optimising resolution and recovery of polypeptides and related macromolecules must accomodate not only the diverse structural vagaries of the solutes themselves, but also their susceptibility to reversible and irreversible deformation of their unique tertiary structures by mobile phase composition or stationary phase characteristics.

Historically, optimisation of resolution in RP-HPLC separations of polypeptides has been approached through the manipulation of mobile phase composition. The effects of the organic solvent modifier[3,8], buffer ions[1,9], temperature[7], flow-rate[3,10] and sample size[4] on the elution behaviour of polypeptides and proteins with a specified generic column have been documented. Criteria for optimal resolution under linear solvent strength elution conditions of partially unfolded polypeptides and proteins with low-pH mobile phases have been established[3,4]. In many cases, these same elution conditions permit high recovery of biological activities in addition to excellent mass recovery, even for very hydrophobic proteins, such as the protein hormone inhibin[11]. Only more recently have the influences on separation performance of stationary phase pore size, surface area, chemical nature of the bonded surface and even the preparation and pretreatment history of the silica matrix been examined[4,5,10,12]. Most RP-HPLC studies with polypeptides up to *ca.* 15,000 daltons have concentrated on 6–10-nm pore diameter silicas, bonded with *n*-alkyl chains differing in chain length up to C_{30} with and without end-capping. Studies with large, denatured polypeptides, such as cyanogen bromide fragments, as well as with some

hydrophobic proteins with molecular weights > 20,000 have suggested that improved chromatographic performance can be obtained by using silica matrices of nominally 30 nm or larger pore diameter. Accurate evaluation of stationary phase effects, and particularly the role of alkylchain length, particle porosity, accessible surface area and ligand surface density has, however, been hampered by the inadequacy of data provided by many vendors on the physical characteristics of the parent silica matrix as well as the generic bonded phase. Moreover, variations by different manufacturers in their bonding technology and column packing procedures have tended to obscure further the stationary phase contribution to solute selectivity and recovery, even when the same commercial source of the silica matrix has apparently been utilised. The present study examines the role played by the critical stationary phase parameters —porosity, ligand density and surface area— in determining relative retention of peptides and proteins with M_r up to 68,000 daltons on well-characterised octadecylsilicas with pore diameters ranging from 7.3 nm to 30 nm prepared under uniform conditions. In associated papers[4,13,14], the dependency on peak shape on these stationary phase characteristics and the dependence of relative retention on alkylchain length are described.

MATERIALS AND METHODS

Chemical and reagents

Acetonitrile was HPLC-grade, obtained from Waters Assoc. (Milford, MA, U.S.A.) or Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Orthophosphoric acid and sodium dihydrogen phosphate were AnalaR grade reagents from BDH (Poole, U.K.). Water was quartz-distilled and deionised by means of a Milli-Q system (Millipore, Bedford, MA, U.S.A.). The source and purification of the polypeptides and proteins used in this study have been described previously[3,9].

Apparatus

All chromatograms were carried out on either a Waters gradient HPLC system, consisting of one or two M6000A solvent delivery systems, a U6K injector, a M450 variable-wavelength system and for gradient elution a M660 solvent programmer, or a Du Pont Model 850 gradient system (Du Pont Instruments, Wilmington, DE, U.S.A.) fitted with a Rheodyne Model 7120 sample injector with a 50-μl sample loop. Sample injections were made with SGE Model 50A syringes (Melbourne, Australia). The pH measurements were performed with a Radiometer PHM-64 meter, equipped with a combination glass electrode. The developmental alkylsilica packings were kindly provided by Drs. J. Schmit and R. W. Stout (Du Pont). Each stationary phase was based on a 6-μm silica, bonded with dimethyloctadecylsilyl (C_{18}) or dimethyloctylsilyl (C_8) groups to maximum surface coverage with endcapping with trimethylsilyl groups. Stainless-steel columns (25 × 0.46 cm) were used throughout, the columns being packed by the slurry-packing procedure. The characteristics of these various silica-based packing materials are given in Table I. The μBondapak C_{18} columns were commercially available from Waters Assoc.

Chromatographic procedures

Bulk solvents and mobile phases were degassed by sonication. The reversed-

TABLE I

CHARACTERISTICS OF THE VARIOUS ALKYLSILICA PACKINGS USED IN THE PRESENT STUDY

Stationary phase	*Surface area* (m^2/g)	*Average pore size* (nm)	*% Carbon*	*Alkylchain coverage* ($\mu mole/m^2$)*
PMS 60-octadecyl	380	7.3**	18.06	2.20
PMS 100-octadecyl	177	10.0***	8.36	2.19
PMS 150-octadecyl	143	13.0***	6.27	2.03
PMS 300-octadecyl	45	30.0***	3.02	3.11
PMS 100-octyl	177	10.0***	4.88	2.87

* Calculated according to the coverage of dimethyloctadecyl- or dimethyloctylsilyl groups without adjustment for the influence of trimethylsilyl-end capping, and based on the nominal surface area. These calculated values may thus overestimate the surface coverage of the *n*-octadecyl chains by *ca.* 10–15% and the surface coverage of the *n*-octyl chains by *ca.* 20 25% although these differences are anticipated to uniformly apply to the various pore size silicas in view of the conditions of their preparation.

** Determined by nitrogen adsorption measurements (B.E.T.).

*** Determined by mercury intrusion measurements.

phase columns were equilibrated to new mobile phase conditions for at least 60 min at a flow-rate of 2 ml/min. All chromatograms were prepared at 20°C. Sample sizes varied between 0.5 and 10 μg of polypeptide material, depending on its optical density. The retention data were calculated by established methods[3] with sodium nitrate to calibrate the column reversed-phase dead-time, t_0. All data points represent the average of triplicate measurements, which agreed within 2%. A peak asymmetry algorithm was used for the calculation of the peak standard deviation (in time units). The *s*-values and ω-values for the various polypeptides were calculated from linear regression analysis of the isocratic data using a Hewlett-Packard 97 calculator. At some organic solvent compositions asymmetric peaks occurred. However, no significant differences in *s* values were observed when the retention times were determined from peak maxima, first moments, or midpoint of the zone on an area basis.

RESULTS AND DISCUSSIONS

Theoretical considerations

The retention of polypeptides in RP-HPLC on *n*-alkylsilica columns arises as a consequence of size exclusion and surface interactive phenomena. In earlier studies, we demonstrated[3,8,9] that changes in retention behaviour due to surface interactions on a specified column with eluents encompassing the organic solvent range of $0 < \psi < 0.8$ could be rationalised in terms of a two-site solvophobic–silanophilic adsorption model. Under these conditions, retention for a polypeptide, *i*, may be given by

$$k' = K_{sec}\frac{V_P}{V_o} + K_{RP}\frac{V_{S,L}}{V_m} + K_P\frac{V_{S,P}}{V_m} \quad (1)$$

where K_{sec}, K_{RP} and K_P are the size exclusion, reversed-phase retention and polar-phase retention distribution coefficients, respectively, V_o and V_p refer to the mobile

phase volumes contained outside and inside the pores, V_m is the total mobile phase volume inside the column (equal to $V_o + V_p$), and $V_{S,L}$ and $V_{S,P}$ are, respectively, the volumes of the accessible solvophobic and silanophilic binding sites of the chemically bonded stationary phase in the column. For binary mobile phases encompassing the organic solvent range $0 < \psi_s < 0.8$, the capacity factor can be expressed by the sum of the capacity factors due to the solvophobic, silanophilic, and size exclusion retention modes and, to a first approximation, can be given by

$$k' = \rho_r k'_w e^{f(-s\psi)} + [\rho_p k'_0 \, Bf(\psi)]^{-1} + \rho_e k'_{sec} \quad (2)$$

where k'_w is the extrapolated value of the capacity factor for neat water, s is the slope of the plot of the logarithmic capacity factor *versus* the volume fraction of organic modifier, ψ_s, over the range where regular reversed phase selectivities occur; k'_0 is the extrapolated capacity factor with the limiting less polar solvent, B is a chromatographic system constant; k'_{sec} is the capacity factor solely due to size exclusion, and the parameters ρ_r, ρ_p and ρ_e are the weighted mole fractions of the solute in each retention mode. At appropriate mobile phase compositions, where the reversed-phase and polar-phase terms cancel, *i.e.* at the minimum of the log k' *versus* ψ_s plot for a certain set of chromatographic conditions, k' becomes equal to k'_{sec}. Separations of polypeptides on alkylsilica columns, under such conditions, essentially by size exclusion phenomena, have been described elsewhere[4,9].

Elution conditions and stationary phase characteristics can be selected in RP-HPLC of polypeptides where the contributions to retention from polar effects are not significant, *i.e.* when either K_p or $V_{S,P}$ in eqn. 1 approach zero. However, from practical considerations it is easier to design stationary phase characteristics such that $V_{S,P}$ is small by making an appropriate choice of the silica matrix, by suitable thermal or pH pre-treatment prior to bonding and by maximally bonding the surface with *n*-alkylsilyl ligands. When $V_{S,P}$ is small and K_P is also small, over a wide range of mobile phase compositions, retention is anticipated to decrease monotonously as the organic solvent content of the eluent increase, up to the limit of solubility. Under such conditions, polypeptides and proteins will exhibit regular reversed-phase selectivity with a dependency of retention on solvent content over the range of chromatographic interest (*i.e.*, $0.5 < k' < 10$), essentially following the empirical relationship.

$$\log k' = \log k'_w - s\psi \quad (3)$$

However, in contrast to simple, low-molecular-weight solutes, the s values for polypeptides and proteins may be very large[3,9] with the consequence that practical isocratic chromatographic separations can only be carried out over a very narrow range of solvent concentrations.

If the effective thickness of the hydrocarbonaceous ligand for a maximally bonded silica-based packing material of pore diameter m, is $d_{L,m}$, the volume of hydrocarbonaceous stationary phase can be related to the total hydrocarbonaceous surface area, $A_{L,m}$, in the column through the relationship

$$V_{S,m} = d_{L,m} \cdot A_{L,m} \quad (4)$$

When non-polar stationary phases are prepared by bonding similar silica matrices with monolayers of the same *n*-alkyl chains to similar levels of ligand density, the change in stationary phases volumes in packings of different porosities, *m* and *n*, can be evaluated from

$$\frac{V_{S,m}}{V_{S,n}} = \frac{d_{L,m}}{d_{L,n}} \cdot \frac{A_{L,m}}{A_{L,n}} \tag{5}$$

Under conditions of reversed-phase selectivity, the capacity factor of a specified polypeptide is given by

$$k' = (V_R - V_{sec})/V_{sec} \tag{6}$$

where V_R and V_{sec} are, respectively, the total retention volume and the retention volume of the solute that is not due to interaction with the hydrocarbonaceous monolayer. Hence, for a given polypeptide separated on two different columns of identical dimensions, but containing alkylsilicas of pore diameters, *m* and *n*, respectively, and eluted with the same mobile phase, the relationship between relative retention and non-polar surface area, A_L, in the columns can be given by

$$\frac{k'_{i,m}}{k'_{i,n}} = \frac{K_{RP,m}}{K_{RP,n}} \cdot \frac{d_{L,m}}{d_{L,n}} \cdot \frac{A_{L,m}}{A_{L,n}} \cdot \frac{V_{sec,n}}{V_{sec,m}} \tag{7}$$

If the organic solvent content, ψ_s, is fixed, the K_{RP} terms for a specified polypeptide and the d_L terms for a specified *n*-alkyl chain will be constant, provided the solvent extraction isotherm for the two phases is also equivalent. Where these assumptions hold, a linear relationship between relative retention and ligand surface area will exist, *i.e.*

$$\frac{k'_{i,m}}{k'_{i,n}} = \frac{A_{L,m}}{A_{L,n}} \cdot \frac{V_{sec,n}}{V_{sec,m}} \tag{8}$$

If all the non-polar surface area, A_L, is not accessible to a polypeptide once inside the pore, due to solute–ligand conformational constraints, arising from ligand chain-length compression, surface tortuosity, solvation, solute molecular shape or other reasons $K_{RP,m}$ will no longer equal $K_{RP,n}$ and eqn. 7 reduces to

$$\frac{k'_{i,m}}{k'_{i,n}} = \frac{f_m A_{L,m}}{f_n A_{L,n}} \cdot \frac{V_{sec,n}}{V_{sec,m}} \tag{9}$$

where *f* is the fractional accessibility factor, which will be both solute- and eluent-dependent. When a polypeptide has complete access to all the bonded non-polar surface, both outside and inside the pore, the value of the *f*-factor will be unity. The magnitude of the *f*-factor will thus influence the reversed-phase component of retention, whilst the molecular volume of the solute will affect the size exclusion component.

The situation can also be envisaged where the nature of the multisite interac-

tions due to the spatial array and proximity of the *n*-alkyl ligands with a polypeptide or protein solute may differ between, for example, small-pore and large pore packing materials. Since partial (or complete) unfolding of many polypeptides and globular proteins can be expected to occur under RP-HPLC conditions, interaction with the non-polar stationary phase inside the pore may occur with solutes which should nominally have been excluded on the basis of a size-exclusion calibration plot of their native forms.

The exposure of sequestered hydrophobic regions may be one consequence of this unfolding process. Multisite interactions between these topographic hydrophobic domains and non-contiguous regions of the non-polar surface inside the pore would be more favoured with small-pore alkylsilicas, *e.g.*, less than 10-nm pore diameter, compared to alkylsilicas with pores of 30 nm or larger, even though the ligand densities (in μmole/m^2) of the supports may be similar.

When the solutes are excluded from the stationary phase pores, *i.e.* when K_{sec} is zero, the value of the *f*-factor will be directly related to the bonded surface area outside the pores. The total bonded non-polar surface area, A_L, is the sum of the bonded surface area inside, A_P, and outside, A_0, the pores, *i.e.*

$$A_L = A_P + A_0 \tag{10}$$

For most stationary phases, including those employed in the present study, $A_P \gg A_0$. However, when a polypeptide or protein reversed-phase distribution coefficient, K_{RP}, is very large, retention of excluded macromolecules mediated solely by the A_0 term may nevertheless be significant, although clearly the sample load capacity will be proportionately impaired. Experimental verification of this behaviour has been observed[13] with large, macromolecular proteins, such as the glycoprotein, thyroglobulin, as well as with other smaller, globular proteins, separated on small-pore alkylsilicas. Specific hindrance factors will tend to limit solute permeability into the pores (but once inside the pore, the solute may have access to the entire pore-surface area) by several discrete effects. The first effect may be understood by a simple geometric argument. If the polypeptide, *i*, with an effective spherical radius, *r*, hits the rim of a pore of radius, *m*, then the effective pore area available to other solute molecules is decreased by the hindrance factor, h_i, such that

$$h_i = \left(1 - \frac{r}{m}\right)^2 \tag{11}$$

The second effect which contributes to surface area hindrance arises from the hydrodynamic drag on the diffusing solute due to the proximity of the pore walls. According to Renkin[15], this effect can be accomodated in an expanded expression for the hindrance factor such that

$$h_i = \left(1 - \frac{r}{m}\right)^2 \left[1 - 2.1\frac{r}{m} + 2.09\left(\frac{r}{m}\right)^3 - 0.95\left(\frac{r}{m}\right)^5\right] \tag{12}$$

Clearly, low-molecular-weight solutes, such as small peptides with small effective radii will exhibit Renkin factors nearer to unity than will large globular proteins where very small Renkin factors may be expected.

The third effect arises from the propensity of many polypeptides or proteins to undergo aggregation, either in solution or at a solid–liquid interface, particularly with low pH water–organic solvent mixtures. This behaviour will result in the solutes exhibiting apparently larger molecular dimensions in solution and altered diffusion constants. As the pore diameters increase, differences between the hindrance factors that relate the entropic restriction for solute entry into the pores and the *f*-factors inside the pores themselves will rapidly become less significant. Obviously, when the pore diameter is very large, *e.g.*, more than 10-fold greater than the solute diameter, all solute molecules will effectively have complete access to the internal surface area of the pores. However, the participation of metastable and/or irreversible states, involving native, partially unfolded and fully unfolded conformers, may still invalidate the assumption that all molecules of a particular solute interact with the stationary phase at the same number and class of binding sites.

Variation in polypeptide retention among different column packings as the organic solvent content is increased

Figs. 1–5 show data obtained with phenylalanine oligomers on the various silica-based packing materials of different pore diameters as the volume fraction of acetonitrile is varied over the range $0 < \psi_s < 0.7$. The primary mobile phase was 10 m*M* sodium dihydrogen phosphate–15 m*M* orthophosphoric acid, pH 2.3. As is evident from these figures, retention for each peptide solute on these five different stationary phases progressively decreased as the organic solvent content was increased up to the limiting solvent composition of $\psi_s = 0.7$. However, the plots of the logarithmic capacity factor, log k' *versus* ψ_s over the range of solvent compositions examined are clearly non-linear. In contrast to earlier data which we have reported[6,9] on the retention behaviour of these same phenylalanine oligomers on μBondapak columns eluted under similar conditions, the present plots of log k' *versus* ψ_s do not exhibit the same pattern of selectivity reversals at high organic solvent content, nor do they

TABLE II

PEPTIDES AND PROTEINS USED IN THE PRESENT STUDY

The one-letter code for the amino acids and the mol.wt. data is as given by M. O. Dayhoff[16].

Solute	*Number of residues*	*Mol.wt.*
F	1	165
FF	2	312
FFF	3	460
FFFF	4	607
FFFFF	5	754
DRVYIHPF (angiotensin II)	8	1106
DRVYIHPFHL (angiotensin I)	10	1417
Hen lysozyme	129	14314
Sperm whale apomyoglobin	153	17199
Bovine serum albumin	582	68000

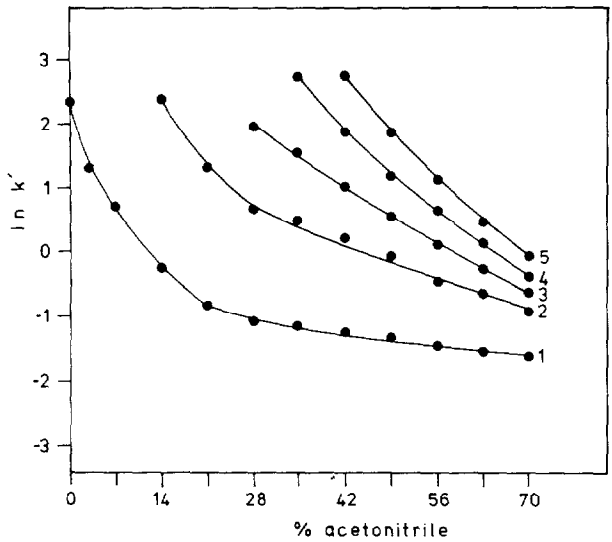


Fig. 1. Plots of the logarithmic capacity factors, ln k', for several phenylalanine oligomers against the volume fraction, ψ_s, of the organic solvent modifier in water–acetonitrile isocratic mobile phases. Conditions: column, octadecyl silica, pore diameter 7.3 nm; flow-rate, 1.0 ml/min; primary mobile phase, 10 mM sodium dihydrogen phosphate–15 mM orthophosphoric acid (pH 2.3) with acetonitrile content adjusted over the range $0 < \psi_s < 0.7$. The peptide key is: 1 = F; 2 = FF; 3 = FFF; 4 = FFFF and 5 = FFFFF. See Tables I and II for other characteristics of the stationary phase and for the one-letter amino acid code.

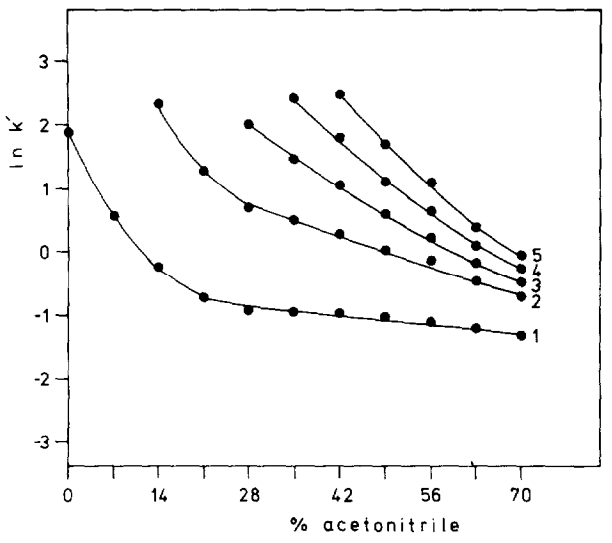


Fig. 2. Plots of ln k' *versus* ψ_s for several phenylalanine oligomers on the 10-nm pore diameter octadecylsilica stationary phase. Other conditions as in the legend to Fig. 1.

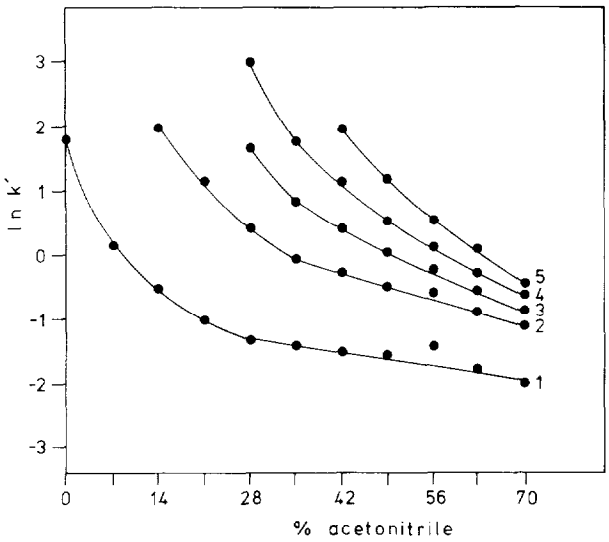


Fig. 3. Plots of ln k' *versus* ψ_s for several phenylalanine oligomers on the 15-nm pore diameter octadecylsilica stationary phase. Other conditions as in the legend to Fig. 1.

show the enhanced retention with water-lean eluents over the range $0.5 < \psi_s < 0.7$, typical of some other types of commercial octadecylsilica supports. A further comparison can be made between these octadecylsilicas and μBondapak C_{18}, namely at the same ψ values the k' values for the phenylalanine oligomers were consistently larger with these new supports, compared to μBondapak C_{18}. Consequently, reversed-phase selectivity is maintained with the four octadecylsilica supports and the

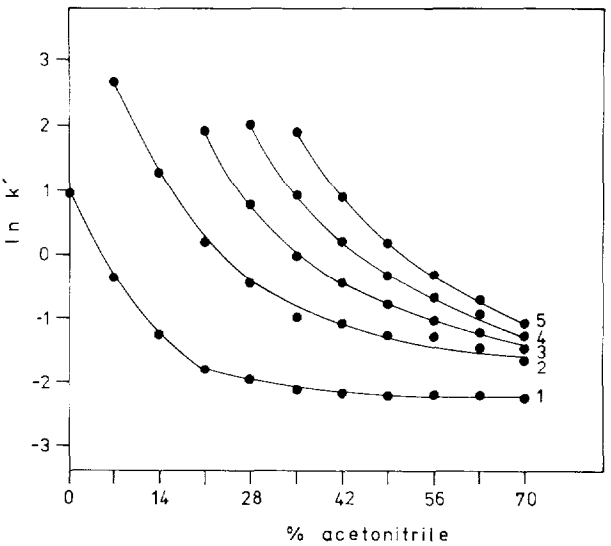


Fig. 4. Plots of ln k' *versus* ψ_s for several phenylalanine oligomers on the 30-nm pore diameter octadecylsilica stationary phase. Other conditions as in the legend to Fig. 1.

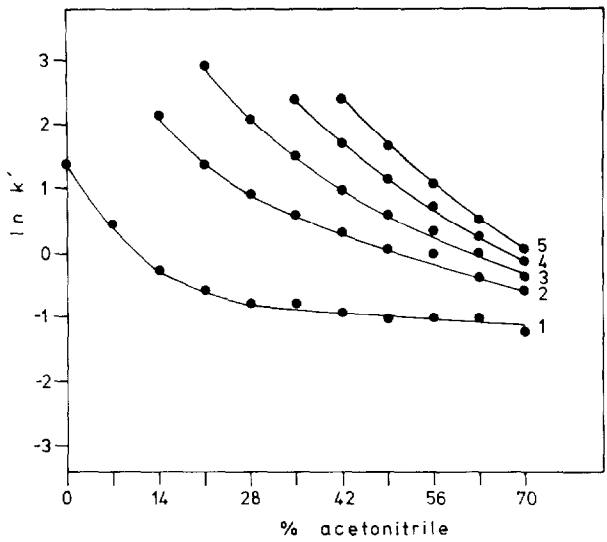


Fig. 5. Plots of ln k' *versus* ψ_s for the several phenylalanine oligomers on the 10-nm octylsilica stationary phase. Other conditions as in the legend to Fig. 1.

octylsilica support used in the present study, even at the final organic solvent concentration of $\psi_s = 0.7$. The apparent loss of selectivity observed with some polypeptides separated on small-pore alkylsilicas with mobile phases encompassing the range $0.5 < \psi_s < 0.8$ has been commented upon previously[1]. Clearly, for the RP-HPLC separation of hydrophobic peptides, it is desirable that high selectivity be maintained with water-lean mobile phases. As is also apparent from Figs. 1–4, the relative retention for a particular peptide on an octadecylsilica at a fixed mobile phase composition decreases as the pore size of the particle increases. Superficially, this decrease in relative retention appears to be correlated with the percent carbon load (Table I). However, as discussed below, relative retention more precisely follows the accessible non-polar surface area in the column. Comparison of the retention data, obtained with the 10-nm pore diameter octadecylsilica and octylsilica supports (Figs. 2 and 5) confirms that the alkyl chain density (in μmole/g) rather than the percent carbon is the dominant parameter.

According to solvophobic theory, selectivity for a homologous series of solutes, eluted under isocratic reversed-phase conditions, is related to mobile phase surface tension, γ, by

$$\tau_{i,j} = \log \alpha_{i,j} = \frac{\Delta(\Delta G_{vdw})_{j,i} + \gamma N(\Delta A_j - \Delta A_i)}{2.3\ RT} \tag{13}$$

where $\Delta(\Delta G_{vdw})_{j,i}$ is the difference in Van der Waals interaction energies for the solutes i and j, $(\Delta A_j - \Delta A_i)$, equal to $\Delta\Delta A_{j,i}$, is the difference in respective molecular contact areas, N is Avogadro's number, R is the gas constant and T is the absolute temperature. Eqn. 13 predicts a linear relation between τ and γ with a slope proportional to $\Delta\Delta A$ for a series of solutes. Figs. 6–10 show the plots of ln k' *versus* mobile phase surface tension, γ, for the oligomeric phenylalanines with binary water–ace-

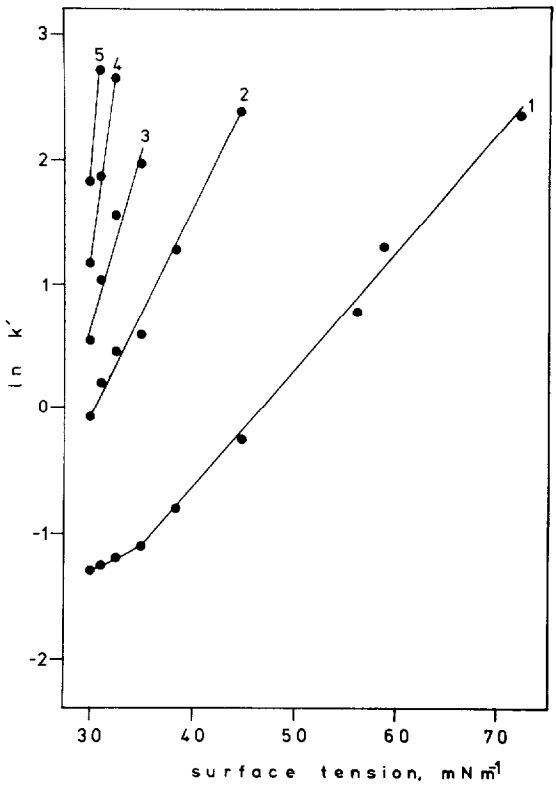


Fig. 6. Plots of the logarithimic capacity factor, ln k', *versus* mobile phase surface tension, γ, for several phenylalanine oligomers on the 7.3-nm pore diameter octadecylsilica stationary phase. Other conditions as in the legend to Fig. 1.

tonitrile eluents. Fig. 11 illustrates the plots of τ *versus* γ for the same series of solutes separated on a μBondapak C_{18} column with similar aqueous methanol and aqueous acetonitrile eluents. Both the plots of ln k' *versus* γ and τ *versus* γ, appear to be consistent with the interpretation that retention of these small peptides on alkylsilicas is a solvent-driven phenomenon, resulting from the expulsion of the solute from the mobile phase. From these experiments, it is possible to calculate the relative magnitude of $\Delta(\Delta G_{vdw})$ from the slopes which give the change in ΔA per phenylalanine residue and from the γ value at τ equal to zero, *i.e.* at the intersection point of the plots of τ *versus* γ which for these solutes on μBondapak C_{18} columns with acetonitrile eluents occurred at γ equal to 29.4 mN/m. This gave a value for $\Delta(\Delta G_{vdw})$ of *ca.* -5.0 J/mole. In other words, the Van der Waals free energy of interaction increases (becomes more negative) by *ca.* 5.0 J/mole as each phenylalanine residue is added to the homologue on a molar basis. It is interesting to compare the overall free energy of association per phenylalanine (Phe) residue with the Van der Waals free energy term per Phe residue. For example, at 8% acetonitrile and 20°C, $\Delta(\Delta G_{assoc})$ for Phe and $(Phe)_2$ equals about -5400 J/mole, while at 32% acetonitrile and 20°C, $\Delta(\Delta G_{assoc})$ equals about -1300 J/mole. Since the free energy of electrostatic interaction should remain essentially constant for pairs of solutes, such as Phe

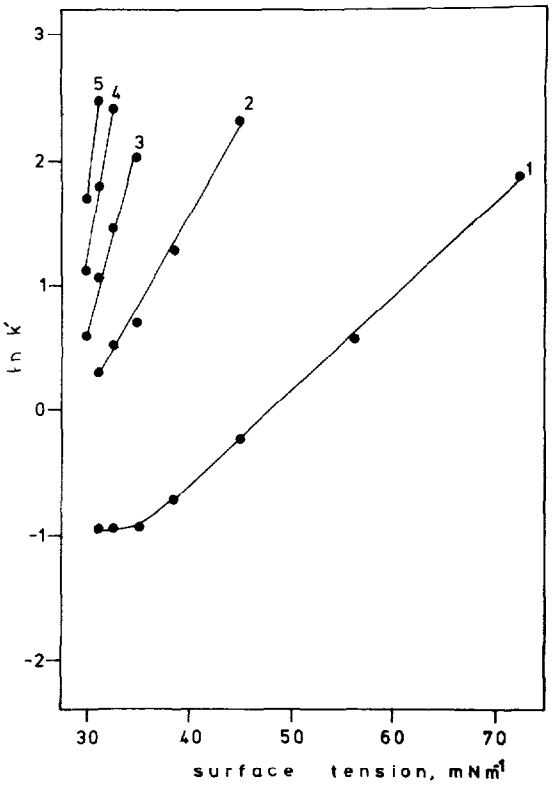


Fig. 7. Plots of ln k' *versus* γ for several phenylalanine oligomers on the 10-nm pore diameter octadecylsilica stationary phase. Other conditions as in the legend to Fig. 6.

and $(Phe)_2$ etc., the greatest contribution to ΔG_{assoc} (and hence to retention) must come from the free energy term responsible for the creation of a cavity in the mobile phase of surface area equal to the solute molecular surface area. Since the radius of the cavity surface for peptides, polypeptides, and proteins is very small (compared to the dimension of the interstitial mobile phase spaces), the surface tension of the cavity will be different to the bulk mobile phase. The curvature correction for converting the macroscopic surface tension to microscopic dimensions is known as the cavity factor, κ^e, and can be related to the capacity factor through the relationship

$$\log k' = C + \gamma \frac{N\Delta A + 4.836\, N^{1/3}\, (\kappa^e - 1)\, V_m^{2/3}}{RT} \tag{14}$$

where ΔA is the contact area between the solute and ligand. When retention is governed by solvophobic interactions, selectivity for two peptides, i and j, separated on the same column under the same isocratic conditions will thus be directly related to $\Delta\Delta A_{i,j}$ and κ^e. It also follows that with stationary phases prepared from the same type of silica matrix, bonded with the same non-polar ligand and having equivalent surface characteristics other than for surface area and pore size the slope of the plots log k' *versus* γ should be constant for a specified peptide, since ΔA and κ^e are constant.

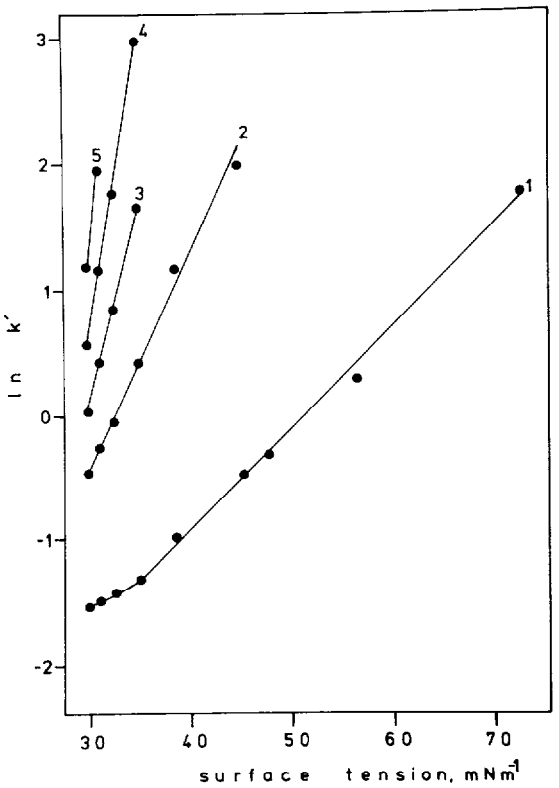


Fig. 8. Plots of the ln k' *versus* γ for several phenylalanine oligomers on the 15-nm pore diameter octadecylsilica stationary phase. Other conditions as in the legend to Fig. 6.

A further corrolary arises from the relationship between ψ_s and γ, namely for peptides separated under reversed-phase conditions the s value of a given peptide should also remain essentially constant with stationary phases of identical surface features except for accessible surface area. According to eqn. 3, linear dependencies of log k' on ψ_s are anticipated. Although, over the wide range of ψ_s examined, linear relationships between log k' and ψ_s for the phenylalanine peptides clearly do not hold, over the range of regular reversed-phase retention, *i.e.* $0.5 < k' < 10$, the calculated s values do exhibit reasonable constancy, taking into account the inherent errors involved. The data summarised in Tables III and IV provide further verification of the retention predictions, based on solvophobic considerations as well as on these empirical relationships. Previous studies[3,4,6] with polypeptides and proteins have demonstrated that the s values, and the ω values increase rapidly with molecular hydrophobicity, the magnitude of these parameters presumably reflecting the number of cooperative topographic binding domains, associated with the adsorption of the solute on the stationary phase surface. The observation that s values and ω values for the phenylalanine oligomers are relatively small (compared to a protein, such as bovine serum albumin where, the s value is > 40) is typical of small peptides and undoubtedly accounts for their generally excellent peak shape and high recovery under appropriate isocratic or gradient-elution conditions.

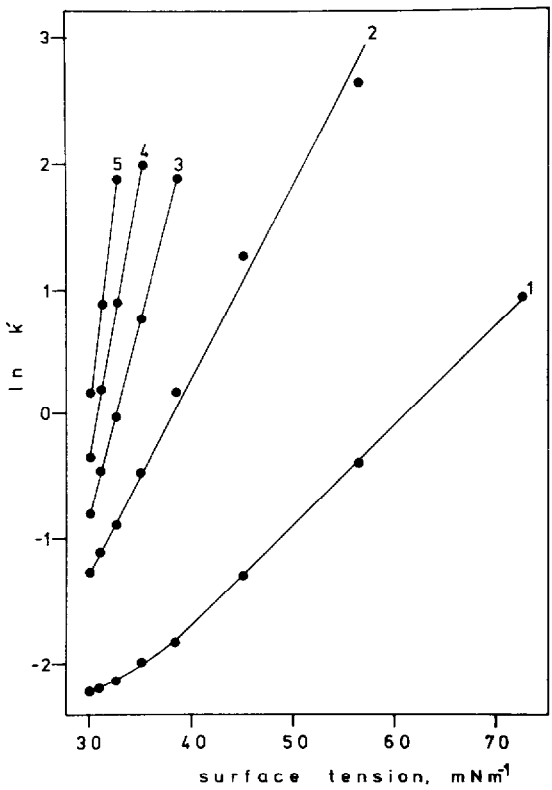


Fig. 9. Plots of ln k' *versus* γ for several phenylalanine oligomers on the 30-nm pore diameter octadecylsilica stationary phase. Other conditions as in the legend to Fig. 6.

Variation of retention with surface area

According to eqn. 9, relative retention for a particular peptide on two different pore size packings will be proportional to the surface area of the bonded alkylsilica. Typical data for the retention of the phenylalanine oligomers obtained at 49% acetonitrile plotted *versus* the corresponding surface area ratio (relative to the 30-nm packing) are shown in Fig. 12. Essentially linear proportionality exists between k' and A_L from the 10-nm to the 30-nm octadecylsilicas, but significant deviation from the expected behaviour is evident for the narrow-pore packing which has the highest surface area. This divergence cannot be accomodated solely in terms of V_{sec} differences. Rather, the data suggest that not all of the non-polar stationary phase is accessible to the solutes with the small-pore octadecylsilica. For all the phenylalanine oligomers, V_{sec} increased as the pore volume increased but the V_{sec} ratio between the 7.3-nm octadecylsilica and the 30-nm octadecylsilica support did not vary by more than a factor of 1.3 for any solute. In addition, the pore structures of the parent matrices used in the present study are generated by the fusion of silica microspheres of narrow size distribution. Hence the pore structure should approximate the single-pore model for permeability, as proposed by Yau and Bly[17]. Evaluation of the Renkin hindrance factor only provides limited information as to whether the entire surface area within the pore is accessible to the solute. For example, according to

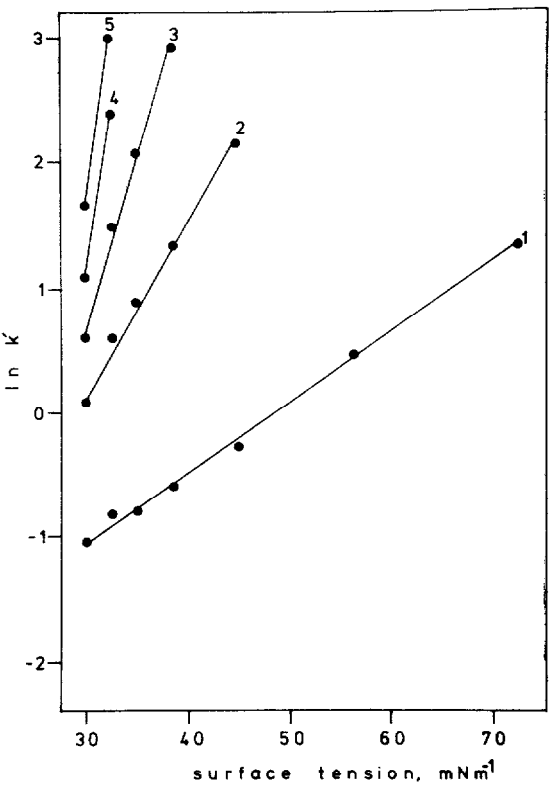


Fig. 10. Plots of ln k' *versus* γ for several phenylalanine oligomers on the 10-nm pore diameter octylsilica stationary phase. Other conditions as in the legend to Fig. 6.

eqn. 12 the hindrance factors for bovine serum albumin with the 7.3- and the 30-nm pore packing are calculated to be 0.0002 and 0.32, respectively. The corresponding hindrance factors for $(Phe)_5$ are 0.18 and 0.71 respectively. When the value of the Renkin hindrance factor of a peptide or protein is very small, as for example, with bovine serum albumin on the 7.3-nm pore diameter packing, then the solute should effectively prevent the entry of all other solutes into the pores, as well as be self-excluding. This would result in very few molecules being inside the pores at any one time. Provided K_{sec} for a peptide or a protein in its native or denaturated state is not zero, interaction with the hydrocarbonaceous ligand within the pore will nevertheless occur. Comparison of dynamic chromatographic data with static sample capacity measurements on alkylsilicas[7,18] indicate that only 5–10% of the stationary phase surface may be utilised in RP-HPLC separations of larger proteins under gradient conditions. If the pore diameter distribution of the stationary phase is large, further restricted access to the total surface area will be anticipated with solutes of different solvated dimensions. The paradoxical behaviour shown by complex protein mixtures, particularly with regard to the influence of sample load on resolution with different pore size packings, may be a direct consequence of these hindrance effects. Further, it is worth noting that the importance of restricted access, as far as limiting multiple

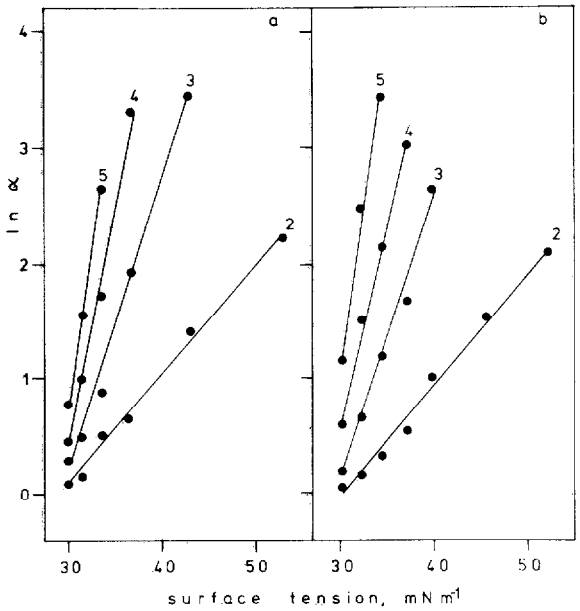


Fig. 11. Plots of the logarithmic selectivity factor, ln α, for several phenylalanine oligomers on a μBondapak C_{18} column. Data shown in (a) correspond to aqueous methanol eluents and (b) to aqueous acetonitrile eluents. Other conditions as in the legend to Fig. 1.

occupancy of a pore surface, will dramatically increase with the molecular size of the solute.

If all of the hydrophobic surface area of the stationary phase was accessible to the solutes, retention would be expected to be proportional not only to the surface area but also to the number of μmoles of non-polar ligand per gram of alkylsilica in the column. For the phenylalanine oligomers, this behaviour is essentially observed

TABLE III

DEPENDENCE OF PEPTIDE ω-VALUE ON MOBILE PHASE COMPOSITION FOR VARIOUS ALKYLSILICA STATIONARY PHASES

Calculated from the plots of log k' *versus* γ over the range $1 < k' < 10$ by regression analysis according to the linear relationship $\log k' = C + \omega\gamma$ where ω is $[N\Delta A + 4.836N^{1/3}(\kappa^e - 1)V_m^{2/3}]/2.3\,RT$.

Stationary phase	*Peptide*				
	F	*FF*	*FFF*	*FFFF*	*FFFFF*
PMS 60 octadecyl	0.08	0.16	0.27	0.60	0.92
PMS 100 octadecyl	0.07	0.15	0.28	0.52	0.80
PMS 150 octadecyl	0.07	0.17	0.32	0.48	0.79
PMS 300 octadecyl	0.07	0.15	0.32	0.47	0.69
μBondapak octadecyl*	0.04	0.13	0.30	0.40	0.57
μBondapak octadecyl	0.04	0.14	0.30	0.50	0.68
PMS 100 octyl	0.05	0.13	0.27	0.53	0.76

* Adjusted with methanol, all other data obtained with acetonitrile.

TABLE IV

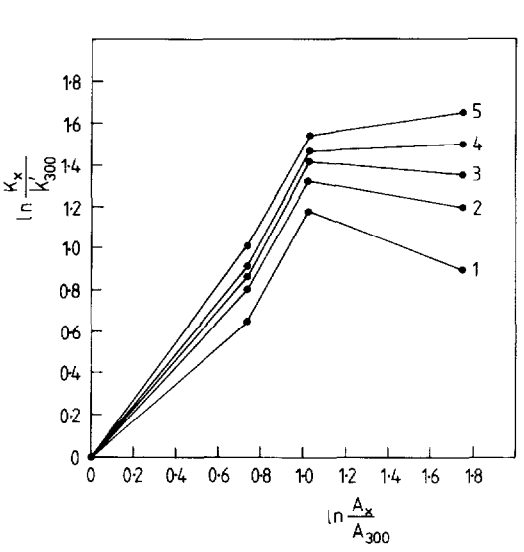
DEPENDENCE OF PEPTIDE s VALUE ON MOBILE PHASE COMPOSITION FOR VARIOUS ALKYLSILICA STATIONARY PHASES

Calculated fom plots of log k' *versus* ψ_s over the range $0.42 < \psi_s < 0.70$ by regression analysis. Correlation coefficients were $r^2 > 0.96$.

Stationary phase	*Peptide*				
	F	*FF*	*FFF*	*FFFF*	*FFFFF*
PMS 60 octadecyl	0.9	3.8	5.7	8.1	10.1
PMS 100 octadecyl	0.8	3.0	5.5	7.9	9.0
PMS 150 octadecyl	0.8	3.0	4.7	6.4	8.1
PMS 300 octadecyl	0.3	2.7	3.6	6.1	8.0
μBondapak octadecyl*	–	5.8	8.3	10.5	12.2
PMS 100 octyl	0.5	3.0	4.8	6.4	8.4

* Data from ref. 3 over the range $0.04 < \psi_s < 0.48$.

(Fig. 13). With the angiotensin peptides (Fig. 14) an increase in retention on the octadecylsilicas from the 30-nm to the 10-nm support is seen, but this then decreases with the 7.3-nm support. Finally, with the proteins apomyoglobin, lysozyme, and bovine serum albumin, retention decreases as the specific ligand capacity increases (Fig. 15). Studies similar to these for the reversed-phase retention of larger globular



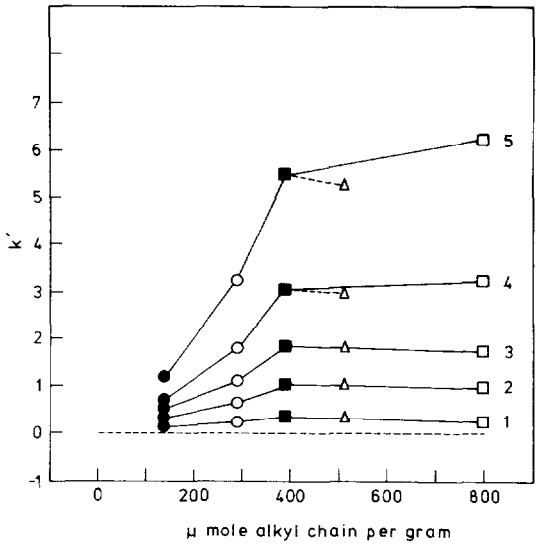


Fig. 12. Plots of relative retention on hydrocarbonaceous surface area of the various octadecyl stationary phases. The data shown were obtained with 49% aqueous acetonitrile, other conditions as in the legend to Fig. 1.

Fig. 13. Plots of the reversed-phase capacity factor *versus* ligand density (in μmole alkyl chain per gram packing material) for the phenylalanine oligomers on porous octadecylsilicas. The data were obtained with 49% aqueous acetonitrile, other conditions as in the legend to Fig. 1. The open triangle corresponds to data for the 10-nm pore diameter octylsilica and the dashed line corresponds to k_{sec} for sodium nitrate.

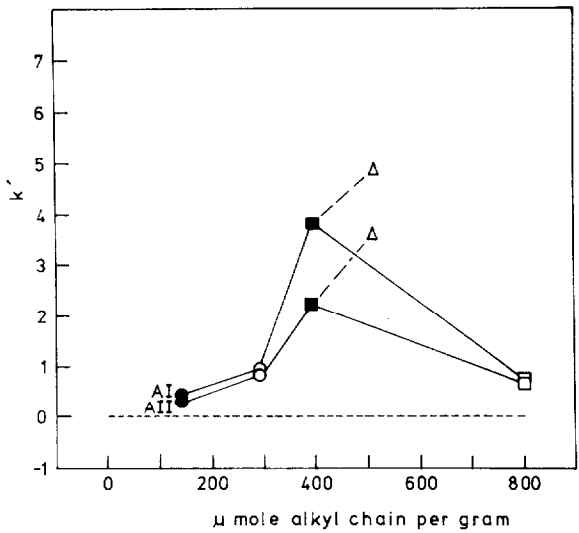


Fig. 14. Plots of the reversed-phase capacity factor *versus* ligand density for the peptides angiotensin I (AI) and angiotensin II (AII) on porous octadecylsilicas. The open triangle corresponds to data for the 10-nm pore diameter octylsilica. The data were obtained with 35% aqueous acetonitrile, other conditions as in the legend to Fig. 1.

proteins have also shown[14] related anomalous ligand capacity effects. It is noteworthy that large polystyrene oligomers also demonstrate decreased reversed-phase retention with small-pore alkylsilicas. For example, Larman *et al.*[19] have shown that polystyrenes with M_r = 50,000 experience an apparent reduction in accessibility to the non-polar surface as the pore size is decreased from 30 to 6 nm, even though K_{sec} for these solutes is *ca.* 0.2. Whether the observed surface area restriction with small pore alkylsilicas is typical of a wider array of proteins and whether it can be attributed to conformational equilibria associated with random coil formation, metastable ad-

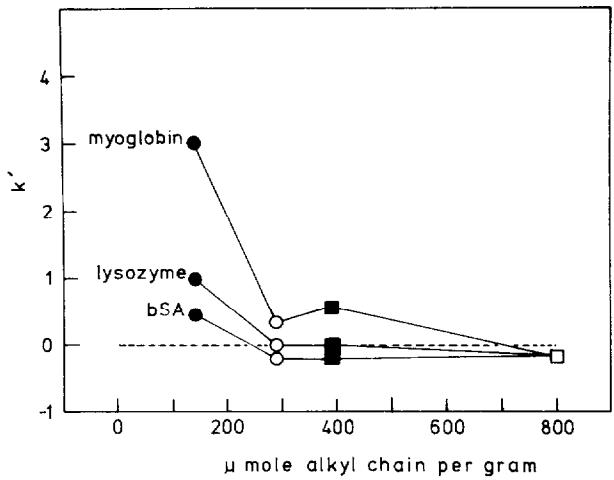


Fig. 15. Plots of the reversed-phase capacity factor *versus* ligand density for sperm whale apomyoglobin, lysozyme and bovine serum albumin on porous octadecylsilicas. The dashed line corresponds to k_{sec} for sodium nitrate. The data were obtained with 49% aqueous acetonitrile, other conditions as in the legend to Fig. 1.

sorption processes, entropic exclusion from the surface monolayer due to crowding effects, solute multilayering at the surface, or other reasons, at this stage remains to be established. However, the data in Figs. 12–15 illustrate that not all the surface area of the non-polar ligand is accessible to polypeptide solutes with narrower pore packings, the extent of the decrease depending on the molecular size of the solute. This latter observation has important ramifications for the determination and comparison of resolution values for proteins on columns packed with alkylsilicas of different pore sizes. For example, claims about the improved performance of a particular non-polar stationary phase for the resolution of proteins must be viewed with caution, unless solutes of similar molecular characteristics are compared.

In summary, the influence of stationary phase porosity and surface area on the retention of peptides and proteins under RP-HPLC conditions has been examined. Comparison of the retention data for the different pore diameter octadecylsilicas has confirmed that solute retention is dominated by solvophobic effects, retention being directly proportional to the surface area of the non-polar stationary phase within the column for small peptides. With larger polypeptides and proteins, anomalous behaviour is evident although the results confirm that size-exclusion phenomena have little effect on the chromatographic retention mediated by the interaction between the non-polar stationary phase and the solute.

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