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

LV*. STUDIES ON THE ORIGIN OF BAND BROADENING OF POLYPEPTIDES AND PROTEINS SEPARATED BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The plate heights, H, and the peak asymmetries, a_s, of several polypeptides and proteins separated on octadecylsilicas within the range of k' values of interest in isocratic or gradient elution optimisation have been investigated. The data indicate that the extent of band broadening for a retarded low molecular weight peptide, eluted at a fixed flow-rate and eluent composition from a small-pore (7.3 nm) alkylsilica, is larger at comparable k' values than observed in the corresponding experiments with a large-pore (30 nm) alkylsilica of similar ligand density, although the differences are not sufficient to result in impaired chromatographic performance. Significant differences are observed, however, when efficiency data for retarded higher-molecular-weight proteins separated on these two different pore size octadecylsilicas are compared. Several factors appear responsible for the changes in H observed as the molecular weight and hydrophobicity of the various solutes were increased. Firstly, slow adsorption–desorption kinetics and associated stationary phase effects appear to contribute significantly to band broadening for retarded polypeptide solutes. These effects are particularly noticeable with proteins whose tertiary structures are not highly stabilised owing to extensive intra- or inter-chain disulphide linkages and other intramolecular forces. Secondly, as the Stokes radius of the solute approaches in magnitude the pore radius of the particle, plate heights for retained solutes appeared to increase more rapidly with the small pore octadecylsilica as the k' is increased than with the larger pore octadecylsilica suggesting that solute diffusion within the particle is decreased. The consequences of these H dependencies on the choice of chromatographic parameters for the separation of polypeptides and proteins on alkylsilicas are examined. In particular, the role of secondary retention effects including protein deformational effects on band dispersion in reversed-phase high-performance liquid chromatography are assessed. The influence of column dimensions and separation time on resolution and recovery in these systems are also

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described. Anomalous behaviour of proteins in reversed-phase chromatography due to slow interconversions between native and partially or fully unfolded forms is discussed in terms of a three-state model.

INTRODUCTION

Traditionally, the chromatographic separation of polypeptides and proteins has been mainly performed on soft polysaccharide permeation gels and ion-exchange resins. However, over the past five years, reversed-phase high-performance liquid chromatography (HPLC) based on rigid, microparticulate, chemically bonded *n*-alkylsilica stationary phases has gained wide popularity for the isolation of peptides and polypeptides from biological extracts[1–5]. A large variety of non-polar stationary and polar mobile phase combinations have been employed in these investigations. Most studies with peptides up to *ca.* 30 amino acid residues have been conducted on porous, microparticulate *n*-octyl- or *n*-octadecylsilica supports with nominal pore diameters in the range 6–10 nm. For the separation of larger polypeptides and proteins, the 5- or 10-μm spherical large-pore (greater than 30 nm) *n*-alkylsilicas have recently been favoured[1,2,6,7]. Generally, the separation of polypeptides and proteins on meso- or macro-porous reversed-phase packing materials requires gradient elution with aqueous–organic solvent mixtures. Ionic and/or non-ionic additives are usually incorporated in the mobile phase to increase solute solubilities and to control the various secondary equilibria manifested by these polyionic solutes in solution and at liquid–solid interfaces. However, even under optimised chromatographic conditions, it is now clearly evident that certain families of polypeptides and proteins can be eluted from commercially available, as well as from the more specialised, laboratory prepared, reversed-phase columns with excellent resolution and almost quantitative recovery, whereas with some other polypeptides and proteins, low recoveries, unsatisfactory resolution and low separation peak capacities are obtained. A further scenario may occur with globular proteins in the sense that resolution and mass recovery may be high but recovery of biological activity may be low, *i.e.* the protein is recovered in denatured form.

Although such chromatographic behaviour can be partly ascribed to composite solvophobic–silanophilic interactions[2,5] between the solutes and the stationary phase, even with well-prepared, maximally bonded, and endcapped *n*-alkylsilica phases, the chromatographic behaviour of polypeptides and proteins remains exquisitely sensitive to small changes in mobile phase conditions or column characteristics. The roles exerted by the organic solvent modifier[8,9], the pH[10] and some ionic and non-ionic modifiers or buffers[1,2,10,11] on polypeptide and protein retention to *n*-alkylsilicas are now well documented. Similarly, the influences of the silica matrix itself and the characteristics of the non-polar stationary phase surface, including the particle porosity, the *n*-alkyl chain length, the ligand density and uniformity of ligand converage, on polypeptides and protein retention have been partially clarified[2,6,7,10,12].

However, the effects of mobile phase and stationary phase parameters on kinetic phenomena associated with polypeptide and protein resolution on *n*-alkylsilicas remain poorly investigated. Several recent studies[8–13] from this laboratory have focused more on the thermodynamic aspects of polypeptide retention in reversed-phase

HPLC under isocratic and gradient elution conditions. The present paper considers, with special reference to kinetic resistance to mass transfer and the ease of diffusion within the stagnant mobile phase in stationary phase pores, several factors associated with the choice of mobile phase composition and stationary phase porosity that affect band spreading of non-sorbed as well as retarded solutes. An associated study examines the influence of elution velocity and column length on band dispersion in the reversed-phase HPLC separation of polypeptides.

EXPERIMENTAL

Equipment

All chromatographic experiments were performed with a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatographic system consisting of two M6000A pumps and a U6K universal injector. The detector used was a M450 variable-wavelength UV detector operating at 210 nm. Most chromatographic measurements were made at 20°C using 25 cm × 4.6 mm I.D. stainless steel columns packed with two developmental octadecylsilicas. The octadecylsilicas had mean particle diameters of 6 μm and specific surface areas of 380 m^2/gm and 45 m^2/gm, with corresponding average pore sizes of 7.3 nm and 30 nm, respectively. Based on carbon analysis the ligand densities of the small- and large-pore stationary phases were 2.2 μmoles dimethyloctadecylsilyl groups per square metre and 3.1 μmoles dimethyloctadecylsilyl groups per square metre, respectively. The μBondapak C_{18} columns were obtained from Waters Assoc. Sample injections were made with SGE Model 50A syringes (SGE, Melbourne, Australia). The pH measurements were performed with a Radiometer PHM-64 meter equipped with a combination glass electrode.

Chemicals and reagents

Acetonitrile (HPLC grade) was obtained from Burdick & Jackson Labs., (Muskegon, MI, U.S.A.). Water was quartz-distilled and deionised using a Milli-Q system (Millipore, Bedford, MA, U.S.A.). The source and purification of the peptides and proteins used in this study have been given previously[9,14]. Orthophosphoric acid and sodium dihydrogen phosphate were AnalaR grade reagents purchased from BDH (Poole, U.K.).

Chromatographic procedures

Bulk solvents and appropriate isocratic mobile phases were degassed by sonication as reported previously[9]. Following a change in eluent composition, the reversed-phase columns were equilibrated to new mobile phase conditions for at least 60 min at a flow-rate of 2 ml/min. Sample sizes varied between 0.5 μg and 10 μg of polypeptide material and were sufficiently small to avoid column overloading. The retention time of the peak at its apex, t_a, was measured in the usual manner. The average elution time of the peak, t_m, and the peak variances, σ_t^2, in units of time were derived[15] from the first and second central moments of the peak. The capacity factors, k', were calculated from the retention times of the solutes which were evaluated at the apex, t_a, or from the centre of gravity, t_m, of the peaks, and $t_{sec(NaNO_3)}$ was chosen as t_o. The effective theoretical plate number, N_{eff}, was evaluated from the t_m and σ_t^2 values, whilst the observed relative theoretical plate number, N_{obs}, was cal-

culated from t_a and peak width at half height, w_h, according to eqn. 5. The peak asymmetry factors, a_s, at 10% of peak height were calculated by established methods.

RESULTS AND DISCUSSION

Theoretical considerations

It is commonly assumed that the retention process for polypeptides and proteins in reversed-phase HPLC involves reversible binding of these polar solute molecules S_1, S_2, ..., S_n to accessible covalently bound ligands, B, at the surface of the stationary phase. This simple process can be expressed as

$$S_i + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} S_i B \tag{1}$$

When no secondary equilibria are involved in the chromatographic process and the condition of linear elution chromatography applies, then the capacity factor, k', the thermodynamic equilibrium constant, K, and the rate constants k_a and k_d for the association and dissociation processes can be inter-related by the equation

$$k' = \varphi K = \varphi k_a \, k_d^{-1} \tag{2}$$

where φ is the phase ratio of the chromatographic system. Under these linear, ideal conditions, the rate of dispersion of the solute band as it migrates along a column can be defined in terms of the effective height equivalent, H_{eff}, to a theoretical plate such that

$$H_{eff} = L \, (\sigma_t^2/t_R^2) = L/N_{eff} \tag{3}$$

where σ_t^2 is the variance of the peak in units of time, t_R is the retention time at the centre of gravity of the peak (equal in the case of gaussian peaks to the retention time, t_a, at the apex of the peak), L is the column length and N_{eff} is the effective theoretical plate number. For skewed peaks the variance of the peak equals $(\sigma_G^2 + \tau_s^2)$ where σ_G is the standard deviation of a Gaussian constituent and τ_s is the time constant of the exponential modifier[16].

If extracolumn plate height contributions are excluded, then the effective reduced plate height, h_{eff}, of an ionised solute, chromatographed on a column of uniform cross-section, uniformly packed with porous spherical particles of mean diameter d_p, and eluted with a non-compressible mobile phase, will be the composite of: (i) axial dispersion of the solute in the bulk mobile phase; (ii) dispersion of the solute arising from transient penetration into the boundary interface and intraparticulate spaces of the porous spherical particles of the stationary phase; and (iii) dispersion arising from the kinetics of interaction of the solute with a qualitatively heterogeneous (*i.e.* solvophobic and silanophilic) lattice of binding sites on the hydrocarbonaceous stationary phase surface. Consequently, h_{eff} may be treated as the summation of the individual plate height contributions which arise from these different processes that independently occur within the column such that

$$h_{eff} = H_{eff}\, d_p^{-1} = h_{kin} + h_{disp} + h_{e,diff} + h_{i,diff} \quad (4)$$

where the kinetic resistance for solute binding at the stationary phase surface is represented by h_{kin}, the longitudinal molecular diffusion and eddy dispersion in the interstitial spaces by h_{disp}, the resistance to mass transfer at the particle boundary by $h_{e,diff}$, and the intraparticulate diffusion resistance to mass transfer by $h_{i,diff}$. The magnitude of H_{eff} (and N_{eff}) will of course be dependent on the flow-rate and the temperature. The experimentally observed reduced plate height, h_{obs}, and the observed relative theoretical plate number, N_{obs}, can be directly calculated by conventional methods assuming gaussian peak shape from the relationship

$$h_{obs} = H_{obs} d_p^{-1} = L d_p^{-1} N_{obs}^{-1} = 0.1803\, L d_p^{-1} w_h^2 t_a^{-2} \quad (5)$$

where w_h is the peak width at half height, provided the mass transfer between the phases is rapid, *i.e.* the peaks are Gaussian. For asymmetrical peaks, calculated H_{obs} values will thus be smaller than H_{eff} values since the former calculations do not take into account peak tailing, *i.e.* the ratio of τ_s/σ_G.

In the most general case, the coupled plate height equation can be written for a particular stationary phase as follows

$$h_{eff} = \frac{B}{v} + f(v_o) + Cv_o \quad (6)$$

where the first term arises from longitudinal molecular diffusion and the third term from slow mass transfer of the solute zone in the stationary phase. The $f(v_o)$ term, which accommodates the effects due to eddy diffusion and slow mass transfer across the moving liquid stream, can, according to Kennedy and Knox[17], be approximated to $Av_o^{1/3}$. Substitution of this approximation into eqn. 6 leads to the well-known Knox equation, *i.e.*

$$h_{eff} = Av_o^{1/3} + B/v + Cv_o \quad (7)$$

As the diffusion coefficient of the solute in the mobile phase, D_m, increases, the reduced velocity of the mobile zone, v_o, given by $Ld_p t_m^{-1} D_m^{-1}$, will decrease for a given particle, column, and linear interstitial mobile zone velocity, u_o. A practical approach to the evaluation of the C-term of eqn. 7 has been derived by Giddings[18] for a bed of packed spheres, namely:

$$C = \frac{1}{30} \frac{k''}{(1 + k'')^2} \frac{D_m}{D_{sz}} \quad (8)$$

where k'' is the zone capacity factor and D_{sz} is the effective diffusion coefficient within the stationary zone. Since k'' is defined by

$$k'' = \frac{\text{mass of solute in stationary zone}}{\text{mass of solute in mobile phase zone}} \quad (9)$$

$$= K\frac{V_{p,i}}{V_o} = K\frac{k_o}{(1 + k_o)} \tag{10}$$

where k_o is the ratio of the intraparticulate void volume ($V_{p,o}$) to the interstitial void volume ($V_{i,o}$) explored by the solute molecules. The term k_o can also be expressed in terms of the appropriate intraparticulate, ε_i, and interstitial, ε_e, porosities such that

$$k_o = \varepsilon_i (1 - \varepsilon_i) \varepsilon_e^{-1} \tag{11}$$

In addition, k' can be related to k'' through the parameter, β, which is the fraction of mobile phase that is stagnant, *i.e.*

$$k' = k'' (1 - \beta) - \beta \tag{12}$$

As the value of k_o is changed for a specific solute through the use of particles of different porosities, relatively small variations in h_{kin} are anticipated, provided the relative retention, u_e, and k_d remain constant. However, large variations in the magnitude of the C-term will become evident with a particular stationary phase when solutes of widely different molecular size are compared, *i.e.* when large variations in k_o and k_d occur.

Temperature and solvent strength changes can affect all the crucial parameters in the plate height equation, particularly through their affect on D_s, D_m and k'. In the range of k' values of interest for resolution optimisation in isocratic or gradient elution reversed-phase HPLC with binary aqueous–organic solvent combinations, *e.g.* over the range $1 < k' < 10$, the dependency of the capacity factor on the volume fraction of the organic solvent modifier, ψ, can, at constant temperature, be approximated by the relationship[8,14,19]

$$\log k' = \log k'_{i,w} - S\psi \tag{13}$$

where S is the solvent strength parameter, which depends on the molecular characteristics of both the organic solvent employed and the solute molecule, and k'_w is the capacity factor of the solute at $\psi = 0$, *i.e.* in pure water. Compared with those of simple solutes, such as benzoic acid derivatives, the S values for polypeptides and proteins are usually large[8,9,14] and generally greater than 20. One consequence of severe log k' *versus* S dependencies, which can be anticipated on the basis of eqns. 5–10, will be significant changes in the magnitude of the incremental contributions to H_{eff} for a polypeptide or protein as the solvent strength of the mobile phase traverses the operational chromatographic range of $1 < k' < 10$. For example, when the log k'_w and S values are large, reversed-phase isocratic elution development is possible only over a very narrow range of solvent strength conditions, a property now well documented[1,2] in the retention/solvent dependencies of many polypeptides and proteins separated on alkylsilicas. Over such narrow elution strength limits, parameters which influence axial dispersion and resistance to mass transfer in or at the boundary of the bulk mobile phase should remain essentially constant at appropriate flow-rates. In addition, over this narrow range of solvent compositions, changes in the phase ratio, φ, of the chromatographic system should also be small. Under these conditions, and at sufficiently high v_o values, the effects of the A term

and the B term of eqn. 7 will be greatly reduced compared with that of the C term, with the consequence that the plate height equation will be dominated by stationary phase contributions and in particular by the h_{kin} term, and the $h_{i,diff}$ term.

Ideally, the h_{kin} term should be evaluated over this k' range from an analysis of the association or dissociation rate constant for each solute and different mobile and stationary phases. However, for complex biomolecules such as polypeptides and proteins, the kinetics of the binding and dissociation processes with alkylsilicas remain largely unexplored. Even if the overall binding phenomenon could be described by such a simple two-state model as indicated by eqn. 1, the actual mechanism and kinetics by which this process is achieved cannot be directly ascertained from the magnitude of the thermodynamic equilibrium constant, K, which corresponds to the free energy change of the process. In turn, the magnitude of the plate height contributions due to the kinetics of association and desorption also cannot be directly determined solely from chromatographic measurements.

Two limiting cases can, however, be envisaged as far as the diffusion contributions to the C term are concerned. Thus, if resistance to diffusion in stagnant mobile-phase pools in intraparticulate spaces is the predominant contribution to h_{eff} for retarded polypeptidic solutes with small-pore n-alkylsilicas, then as the particle porosity is increased, the $h_{i,diff}$ term in eqn. 4 should become less significant with concomitant improvement in efficiency, provided k_a and k_d remain constant for both the small- and large-pore non-polar stationary phases. On the other hand, if resistance to diffusion associated with solute residence in the non-polar ligand monolayer is the predominant contribution to h_{eff}, little variation in plate height and peak asymmetry is anticipated for a polypeptide separated at comparable relative retention on a small- or large-pore alkylsilica, provided the alkyl chain length is the same, the ligand densities are similar, the columns have identical dimensions and other chromatographic parameters, such as flow-rate, temperature, etc., are held constant. If we assume that the hydrocarbonaceous stationary phase is composed of uniform porous spherical particles, then the reduced plate height arising from these kinetic processes can be evaluated, according to the treatments of Giddings[18] and Knox[20], by

$$h_{kin} = \frac{2\gamma}{\nu} + A\nu^{1/3} + \frac{1}{30}\frac{k''}{(1 + k'')^2}\frac{D_m}{D_{sz}}\nu \qquad (13)$$

where γ is the obstruction factor. Hence, even in the absence of detailed isotherm data and kinetic data on the rate constants associated with adsorption and desorption of polypeptides and proteins to/from n-alkylsilicas, it is nevertheless possible to evaluate qualitatively the importance of the contributing parameters to the plate height from comparisons of the dependencies of peak efficiency and peak asymmetry on relative retention for a selection of small peptides and globular proteins separated on alkylsilicas under isocratic conditions. In addition, these comparisons provide an experimental framework to test the validity of the assumptions made about the retention process itself.

Variation in plate height and peak asymmetry for small peptides and proteins separated on octadecylsilica columns as retention is increased

During the course of this investigation, chromatographic separations of a fam-

ily of phenylalanine oligomers, Phe to $(Phe)_5$, angiotensin- and insulin-related polypeptides, and several small globular proteins (Table I) were carried out on columns of identical dimensions, packed with two different 6-μm octadecylsilica stationary phases of similar ligand coverage. The average pore diameters of the two silica-based supports were 7.3 nm and 30 nm. Isocratic elution was achieved by using aqueous 30 m*M* sodium dihydrogen phosphate–15 m*M* orthophosphoric acid, pH 2.1, containing various percentages of acetonitrile. The organic solvent content for the different isocratic experiments was varied sequentially so that, where possible, the relative retentions for the various peptides and proteins were consistent with optimal reversed-phase elution behaviour; *i.e.* within the range $1 < k' < 15$ and k' values decreased as the organic solvent volume fraction, ψ, increased. Previously, we have investigated[9,14] the chromatographic behaviour of these same solutes under gradient elution conditions of the same primary buffer together with a variety of 0–50% acetonitrile linear gradient programs. Under these gradient elution conditions all of these solutes gave reproducible gradient retention times, high recoveries (at least 90%) and narrow bandwidths. These observations suggest that secondary retention processes have been adequately controlled by the choice of this mobile phase composition, at least as far as gradient elution is concerned. Table II lists representative data for the capacity factors and plate heights for several polypeptide solutes, evaluated at the apex, t_a, or from the centre of gravity, t_m, of the peaks. Although the calculations for H_{obs}, based on peak width at half height, w_h, did not take into account peak tailing, the general trends evident with H_{eff} discussed below were also followed by H_{obs}. In common with other reports[15,21,22] on the influence of peak skew on plate height calculations, the calculations based on w_h underestimated the plate height, *i.e.* for all the peptidic solutes investigated the value of H_{obs} was less than H_{eff}. As also noted in other studies[15,28] with low-molecular-weight solutes, peak

TABLE I

PEPTIDES AND PROTEINS USED IN THE PRESENT STUDY

The one-letter code for the amino acids is as suggested by M. O. Dayhoff in *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, MD, 1968.

No.	*Solute*	*Numberof residues*	*M.W.*
1	Phenylalanine (F)	1	165
2	Diphenylalanine (FF)	2	312
3	Triphenylalanine (FFF)	3	460
4	Tetraphenylalanine (FFFF)	4	607
5	Pentaphenylalanine (FFFFF)	5	754
6	Angiotensin III (RVYIHPF)	7	974
7	Angiotensin II (DRVYIHPF)	8	1106
8	Angiotensin I (DRVYIHPFHL)	10	1417
9	Bovine insulin B chain	30	3401
10	Bovine insulin	51	5782
11	Hen lysozyme	129	14,314
12	Sperm whale apomyoglobin	153	17,200
13	Porcine trypsin	223	23,300
14	Bovine serum albumin	582	68,000

efficiencies for the small peptides improved slightly under a particular set of isocratic mobile phase conditions from the earlier to the later eluted peptides. Because of the limited elution strength range over which the polypeptides and proteins examined in the present study could be chromatographed on the alkylsilicas, it was not possible to establish whether similar behaviour patterns occur with all of the larger solutes. Such comparison should however be possible with homologous protein variants. Furthermore, in all cases examined, as the retention of a specific peptide or protein was progressively increased by decreasing the acetonitrile content of the mobile phase, the N values of these solutes decreased, and the corresponding H values increased. With the small polypeptides, these values appear to approach asymptotic limits, the magnitude of which was different for each solute.

Data obtained from these experiments with the phenylalanine oligomers, separated on the two different pore size octadecylsilicas, are shown in Fig. 1. Because of the narrowness of the pores in the 7.3-nm pore diameter octadecylsilica, it is possible that the mobile phase layer close to the intraparticulate surfaces of the sta-

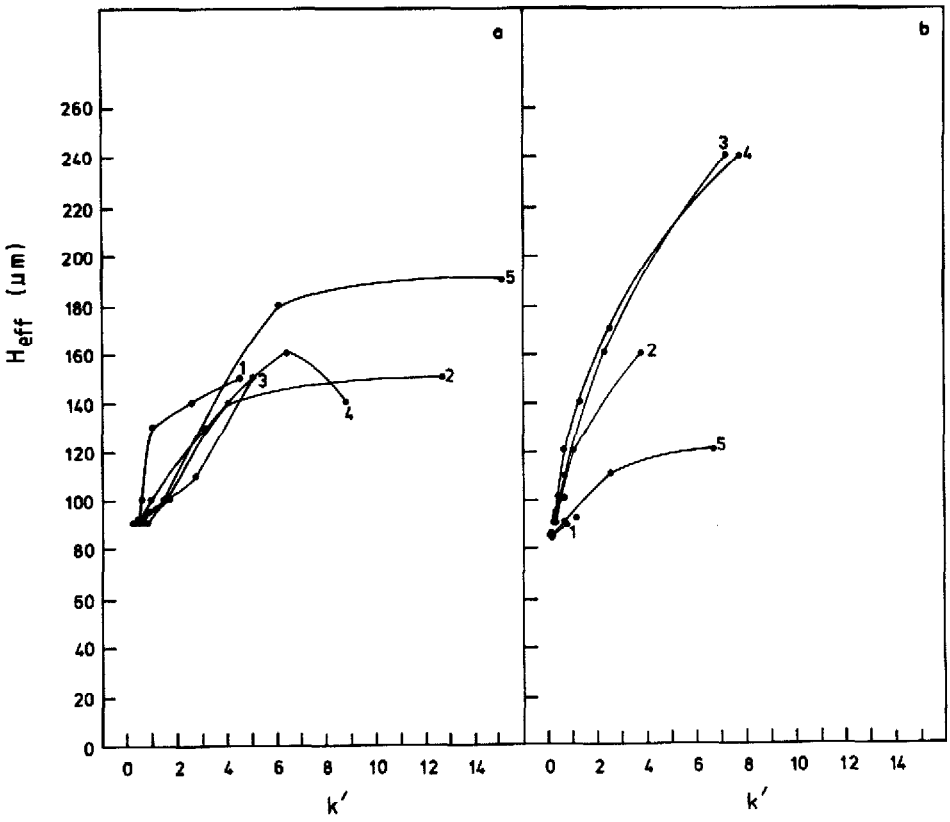

Fig. 1. Effect of mobile phase strength on H_{eff} for small peptides separated on a 7.3-nm pore size (a) or a 30-nm pore size (b) octadecylsilica at 2.0 ml/min. The mobile phase consisted of 30 mM sodium dihydrogen phosphate and 15 mM orthophosphoric acid, pH 2.1, containing various percentages of acetonitrile such that relative retention encompassed the range k' 0–10. The peptide key is: 1 = F; 2 = FF; 3 = FFF; 4 = FFFF; 5 = FFFFF. See Table I for the code to the one-letter abbreviations and molecular weights.

TABLE II

REPRESENTATIVE DATA ON PLATE HEIGHTS AND PEAK SYMMETRIES

Calculated according to peak moment or peak maxima methods for several polypeptides and proteins, separated on *n*-alkylsilicas of different porosities

*Solute**	*Octadecylsilica (7.3-nm pore diameter)*						*Octadecylsilica (30-nm pore diameter)*					
	k'	N_{obs}*	N_{eff}**	H_{obs}**,§	H_{eff}***,§	*Peak asymmetry, a_s*	k'	N_{obs}**	N_{eff}***	H_{obs}**,§	H_{eff}***,§	*Peak asymmetry, a_s*
3	0.45	3950	2760	63	91	1.33	0.45	3300	2450	76	100	1.54
	5.10	2250	1660	110	150	2.50	7.11	1430	1060	170	240	2.50
5	0.82	3690	2740	68	91	1.43	0.68	3750	2780	67	90	1.67
	15.10	1790	1290	140	190	2.50	6.70	2750	2040	90	120	1.92
8	0.01	890	670	280	370	1.27	0.05	2220	1650	110	150	1.67
	9.17	610	507	410	490	2.44	5.40	290	250	860	1000	3.33
10	−0.15	2250	1590	110	160	1.54	0.05	1420	1010	180	250	1.43
	12.2	100	110	2500	2270	4.35	0.75	260	220	960	1140	2.68
11	−0.17	1450	1040	170	240	1.43	0.08	1350	1000	190	250	1.52
	0.45	30	20	8330	12500	6.67	0.48	159	140	1570	1790	2.22
13	−0.16	1720	1200	150	210	1.82	−0.04	1690	1290	150	190	1.67
	0.05	35	20	7140	12500	8.33	0.60	140	140	1790	1790	3.33
14	−0.18	1410	1030	180	240	1.39	−0.12	1410	1010	180	250	1.61
	0.86	50	40	5000	5680	10.0	0.64	70	50	3570	5000	4.35

* See Table I for key to solutes.

** Calculated according to eqn. 7 from retention time at peak apex and peak width at half height.

*** Calculated according to peak moment method.

§ Values of H_{obs} and H_{eff} are in μm.

tionary phase is highly structured and that solute diffusion within this layer, as well as in the non-polar ligand monolayer itself, is slow. When the diffusion of the solute within the pore is restricted, *i.e.* in circumstances where effective diffusion coefficients in the stationary phase monolayer, D_{sz}, or in the adjacent stagnant mobile phase regions, D_i, are less than D_m, mass transfer into and out of the particle will also be reduced with the consequence that the C term will be proportionately increased. Mobile phase diffusion coefficients, D_m, of macromolecular solutes have been found[23,25] to be six to fifty times larger than the solute diffusion coefficient within porous materials. This difference can be contrasted with the chromatographic behaviour of small molecule solutes, where Horváth[26] has concluded that D_s and D_m are of comparable value, whilst Stout *et al.*[27] have found that D_s is roughly one half as large as D_m in reversed-phase systems with phthalate esters. Recent unpublished work from Snyder *et al.* indicates that D_s is approximately one tenth of D_m for small peptides in a typical reversed-phase HPLC system. Since the diffusivities in water of small peptidic solutes with molecular weights up to *ca.* 600 are in the range $1 \cdot 10^{-5}$ cm^2/sec to $5 \cdot 10^{-5}$ cm^2/sec whilst with polypeptides and proteins their diffusivities are one to two orders of magnitude lower, there will be significant differences in

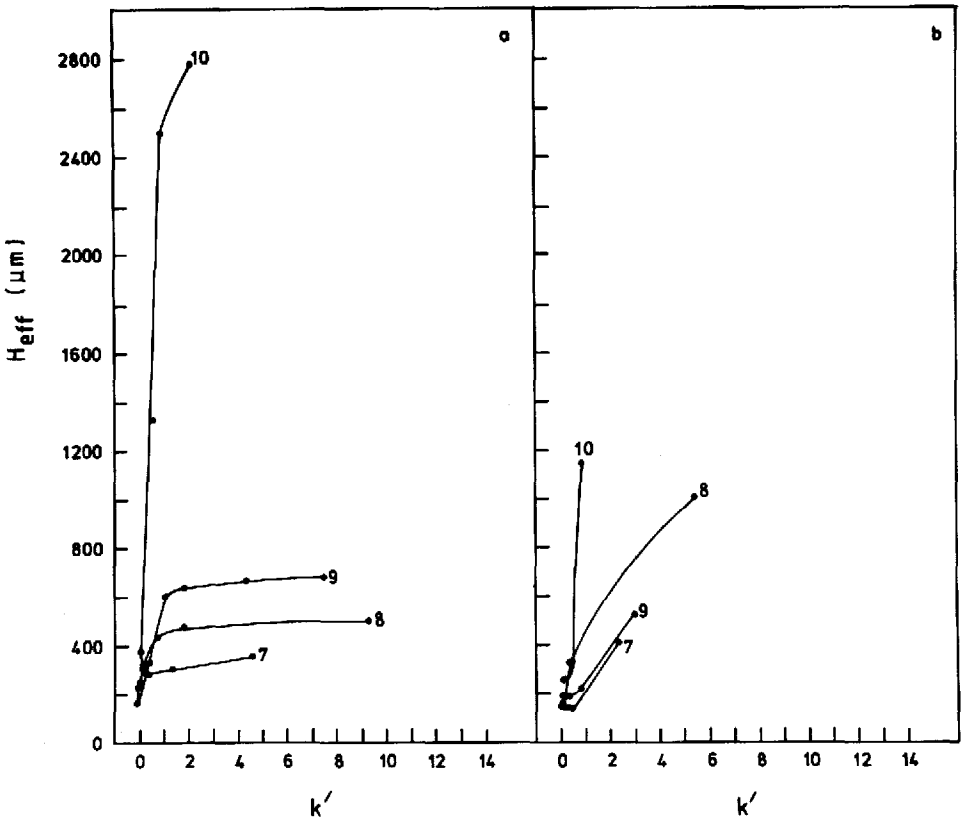

Fig. 2. Effect of mobile phase strength on H_{eff} for several polypeptides and proteins separated on a 7.3-nm pore size (a) or a 30-nm pore size (b) octadecylsilica at 2.0 ml/min. The polypeptide and protein key is: 7 = angiotensin II; 8 = angiotensin I; 9 = bovine insulin B chain; 10 = bovine insulin. The chromatographic conditions are the same as in the legend to Fig. 1.

stationary phase and mobile phase contributions to h_{eff} as the molecular weight of the solutes increases. It is worth recalling here that only diffusion in the mobile phase contributes to the A term of the plate height equation, whilst the C term involves diffusion contributions from stagnant mobile phase and stationary phase effects. Comparison of the efficiency data for each of the phenylalanine oligomers, separated at the same mobile phase composition and linear velocity on the 7.3-nm and the 30-nm pore diameter octadecylsilica suggest that the respective diffusivities of these small solutes and their rate constants with these two stationary phases are of similar magnitude but not identical. The overall change in H for an individual phenylalanine oligomer, chromatographed on either stationary phases, was by a factor of *ca.* 2 as the retention of the solute was increased within the range $1 < k' < 10$. The angiotensin- and insulin-related polypeptides exhibited similar, but more pronounced changes in peak efficiency, a larger change in N_{eff} of H_{eff} with increasing k' being evident with several peptides, *e.g.* angiotensin I the 30-nm pore diameter octadecylsilica (Fig. 2). In a previous study on the variation of polypeptide retention on nonpolar stationary phases of different porosities but similar ligand densities, we demonstrated[12] that pore size effects and associated with restricted steric access can occur with polypeptides as small as the angiotensins when small-pore alkylsilicas are used. The observed changes in H for the angiotensin- and insulin-related polypeptides are

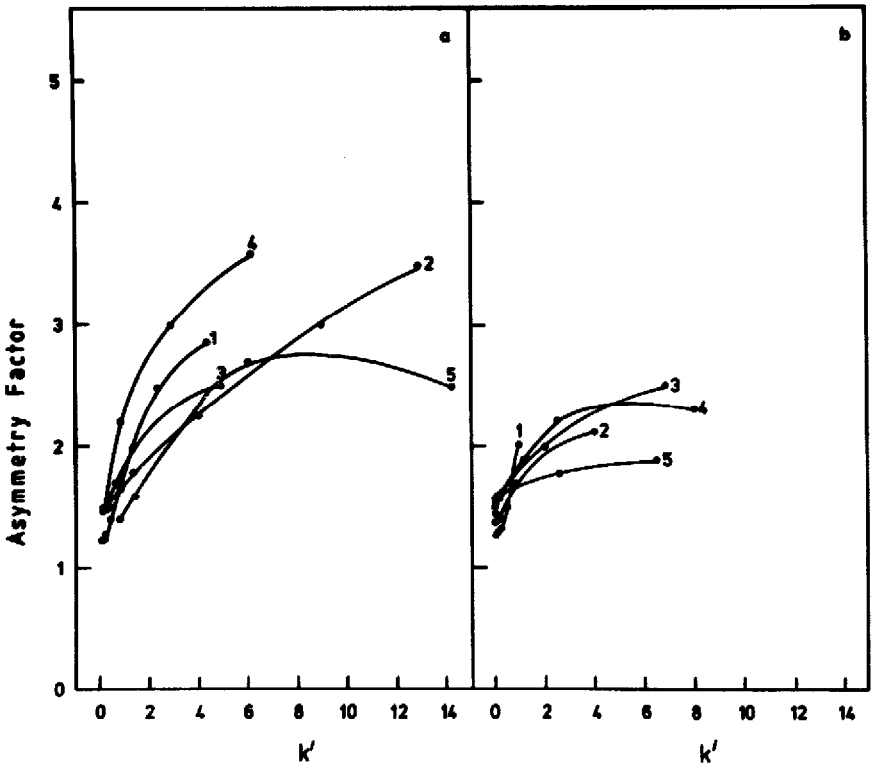

Fig. 3. Effect of mobile phase strength on peak asymmetry, a_s, for several small peptides. The chromatographic conditions and peptides key are the same as in the legend to Fig. 1.

consistent with this chromatographic behaviour, although the origin of the apparent biphasic variation in H, most noticeable with the 7.3-nm support, is unclear.

For each of these small peptidic solutes, the asymmetry factors, a_s, were greater than unity and progressively increased towards an (apparently asymtotically) limiting value, as relative retention was systematically increased over an elution range compatible with the reversed-phase operational requirements (Figs. 3 and 4).

Efficiency and peak asymmetry data from corresponding experiments with several larger polypeptides and small globular proteins are shown in Figs. 5 and 6. Compared with the data described above for the small peptides, the increases in band dispersion observed for these larger solutes, such as porcine trypsin, were much more dramatic as the relative retention was increased, even in circumstances where the change in k' value for a particular polypeptide or protein between two different isocratic measurements was very small, *e.g.* less than 0.2. These changes in H were also associated with significant decreases in peak symmetry (Fig. 6). Although the changes in peak asymmetries for porcine insulin and the insulin B-chain polypeptide were similar to those observed with the small peptides over comparable k' retention ranges, the increases in the asymmetry factors for the small proteins investigated were particularly striking. In practical terms, similar significant increases in band dispersion induced by very small changes in mobile phase compostion —and the associated pronounced variations in retention— result in considerable difficulty in optimising

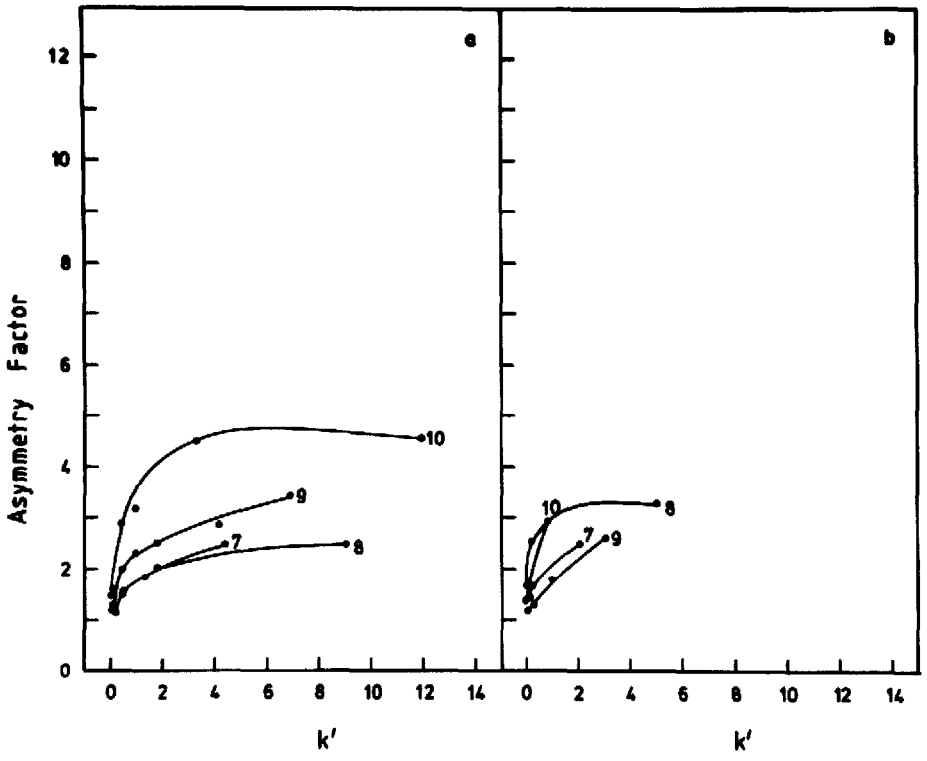

Fig. 4. Effect of mobile phase strength on peak asymmetry, a_s, for several angiontensin- and insulin-related polypeptides. The chromatographic conditions and solute key are the same as in the legend to Fig. 2.

resolution for even closely related polypeptides above *ca.* 30 amino acid residues in length under isocratic elution conditions. However, for smaller peptides, isocratic separation with good peak shape can be achieved provided the mobile phase composition is chosen to ensure the k' value of the last-eluted peak is less than *ca.* 10. A typical example of an isocratic separation of several angiotensin-related peptides under these conditions is shown in Fig. 7.

Large pore diameter silicas (*i.e.* with nominal pore diameters greater than 15 nm) have gained popularity recently for the separation of large polypeptides and proteins[6,7,29–31]. In these earlier investigations, comparison of band dispersion of a solute on a small- or large-pore alkylsilica has usually been based on gradient elution data under the assumption that useful information on efficiencies can be directly calculated from equations developed for isocratic linear elution chromatography. The data shown in Figs. 1–6, albeit limited in polypeptide or protein variety, demonstrate that the band dispersion processes, as expressed by the plate heights and peak asymmetries determined from isocratic measurements, are much more complex than may be evident from gradient elution experiments irrespective of whether al-

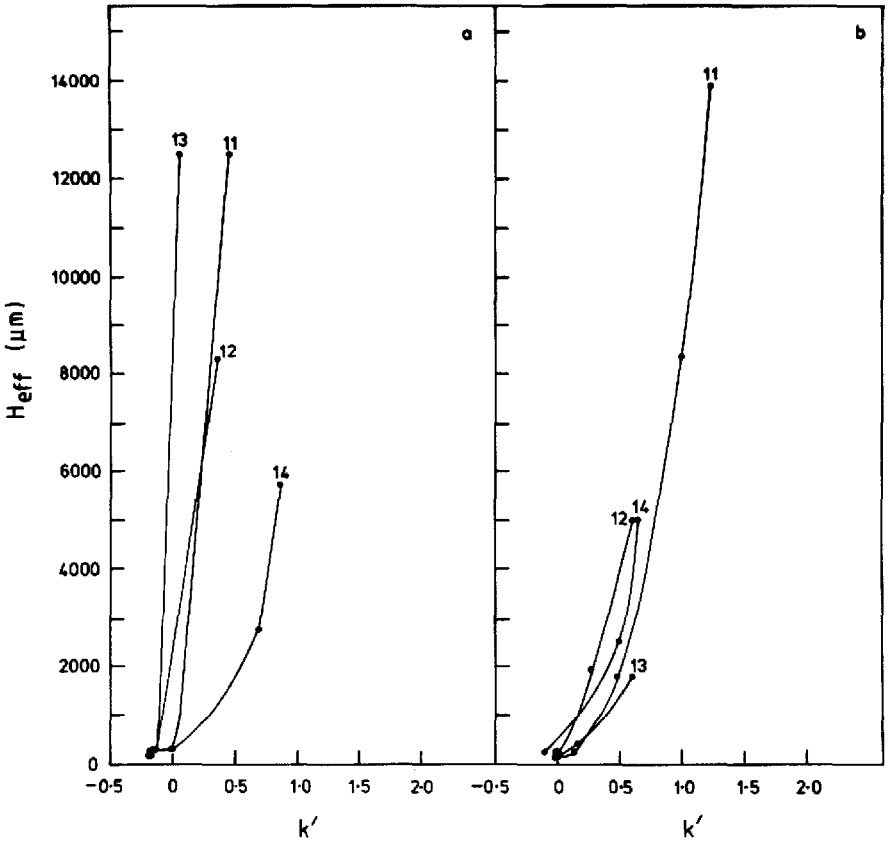

Fig. 5. Effect of mobile phase strength on H_{eff} for several polypeptides and proteins separated on a 7.3-nm pore size (a) or a 30-nm porse size (b) octadecylsilica at 2.0 ml/min. The polypeptide and protein key is: 11 = hen lysozyme; 12 = sperm whale apomyoglobin; 13 = porcine trypsin; 14 = bovine serum albumin. The chromatographic conditions are the same as in the legend to Fig. 1.

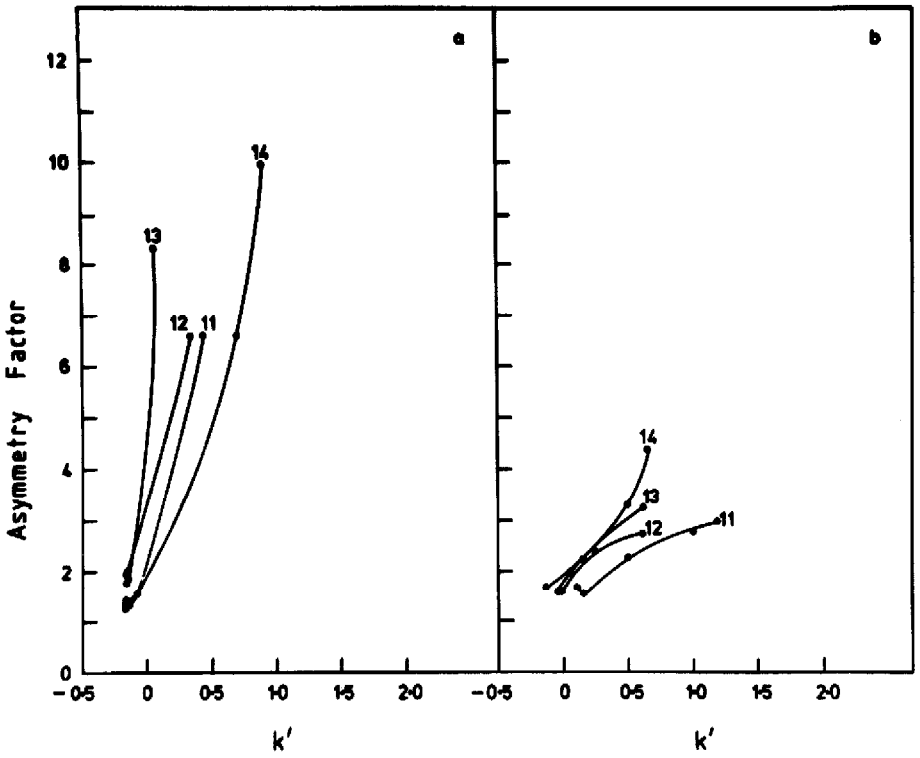

Fig. 6. Effect of mobile phase strength on peak asymmetry, a_s, for several polypeptides and proteins. The chromatographic conditions and solute key are the same as in the legend to Fig. 5.

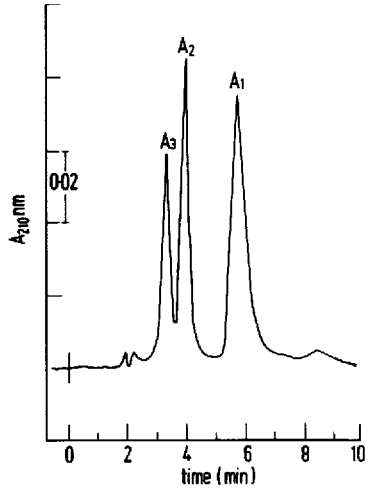

Fig. 7. Separation of several angiotensin peptides under isocratic elution conditions. Chromatographic conditions: column, μBondapak C_{18}; flow-rate, 2.0 ml/min; mobile phase, 15% acetonitrile in aqueous 0.1% orthophosphoric acid. Peptide key: 1 = angiotensin III; 2 = angiotensin II; 3 = angiotensin I.

kylsilicas of different porosities are employed. The conclusion can also be drawn from these data that the improved resolution noted with some large-pore alkylsilicas should probably be more accurately ascribed to differences in relative selectivity of the stationary phase rather than to the plate height of a particular column. In fact, variation in the source and treatment of the silica matrix itself, and the procedures used for the chemical modification of the surface, have already been implicated[7,12] in these resolution differences without the need to invoke the involvement of specific effects due to porosity differences. Further, studies[13,32] with several 'standard' proteins and a variety of small- and large-pore alkylsilicas have suggested that as little as 5% of the total stationary phase surface area may actually be involved in interaction with ionised peptides or proteins under regular reversed-phase conditions. Clearly, further detailed investigations are required if the current complex and controversial issues of the relationship between restricted diffusion and pore structure of the particle, surface tortuosity and accessibility of the non-polar surface area as the pore diameter is changed are to be adequately understood. However, the observed increases in plate heights when the small-pore octadecylsilica was used with the polypeptides and proteins listed in Table I do have precedences. For example, increases in plate height presumably due to an increase in the C term, for retained small molecule solutes have been observed by Engelhardt *et al.*[33] and by Stout *et al.*[27] with stationary phases of pore diameters less than 10 nm.

The practical implications of the data summaries in Figs. 1–6 are very important. Firstly, as isocratic mobile phase elution strengths are decreased, *i.e.* as the relative retention of the solute increases, H values for polypeptides and proteins increase rapidly. Similar k'- and flow-rate-dependent changes in H and a_s have already been noted[23,34,35] in affinity and ion-exchange chromatography, where the h_{kin} term also plays a significant role in peak dispersion. Secondly, in order to minimise band dispersion, isocratic elution conditions must be selected so that secondary retention effects as well as solute conformational effects are controlled. Secondary retention effects arising from heterogeneity of the stationary phase binding sites can frequently be controlled for small peptides by appropriate choice of mobile phase composition, thus ensuring minimal H values even when large k' values are involved. High ionic strength, low pH conditions in the presence and absence of cationic reagents, such as tri- or tetra-alkylammonium salts, have commonly been used[1,2,5] to eliminate this type of secondary retention effect. However, conditions similar to these are known to induce also significant conformation changes in polypeptide secondary and tertiary structure leading to random coil formation. For small polypeptides these conformational changes may not be deleterious as far as recovery of biological function is concerned but with many proteins such changes often result in irreversible unfolding. As a consequence, a compromise between optimal mobile phase composition as far as control over secondary retention effects and conformational effects must be reached. In some cases it may be possible to select elution conditions from the log k' *versus* ψ plots so that the mobile phase composition corresponds to a region of appropriate k' values for the substance of interest. Even when a narrow bandwidth is achieved for a particular peptide or protein chromatographed on an alkylsilica stationary phase, the elution condition could still result in the recovered component being biologically inactive. Thirdly, the data on the changes in H, and a_s, with retentions obtained from isocratic measurements, in essence indicate the limits in band

dispersion which can be expected for widely dissimilar gradient shapes of the same primary buffer composition and the final organic solvent modifier composition. Very shallow gradients at low flow-rates, where band compression factors, G, are relatively small, can result[10,29,30] in poor peak shape and poor recovery for strongly retained polypeptides and proteins, particularly when the initial organic solvent modifier conditions are inappropriately distant from the solvent concentrations at which desorption occurs. Under such conditions, the solute dwells at the head of the reversed-phase column for a considerable time. This dwell or residence time, t_w, can affect[36] bandwidth, retention and recovery of some proteins through, as yet, uncharacterised mechanisms. One possibility, which we believe may be important in this regard, involves surface-induced unfolding of native proteins. When the initial solvent strength limit is close to the elution concentration, when the dwell time is small, and when the optimal value of the gradient steepness parameter, b, is achieved, it has been our experience that detection sensitivity, separation speed, resolution and, frequently, mass and biological recovery of globular proteins up to *ca.* 80,000 daltons all improve! Finally, in situations where isocratic elution is possible, *e.g.* in the separation of a series of peptidic analogues, the efficiency data can be used to quantify column performance as part of the experimental choice whether a long column (*i.e.* >15 cm) with a stronger eluent or a short column (*i.e.* <5 cm) with a wider range of relative retention is the more suitable option. In these situations the effective peak number, n, provides a useful measure of resolution of such chromatographic systems. According to Guiochon[37], the peak number can be approximated by

$$n = \frac{\sqrt{t_o}}{4}\sqrt{\frac{u_e}{H}}\ln\frac{t_R}{t_o} \tag{14}$$

Hence the number of peptidic components in a mixture that can be theoretically eluted in an analysis time, t_R, with a resolution of unity will increase with the square root of u_e/H, assuming that u_e and H are constant for all solutes, *i.e.* provided k_o, and h_{kin}, etc., are the same for all solutes.

Influence of protein deformation on band dispersion in reversed-phase HPLC

The reversible and non-reversible deformation of proteins in water/aqueous–organic solvent combinations and at liquid–solid interfaces is well documented. Previous studies from this and other laboratories have concluded[1,2,5,8,12,38] that the adsorption of polypeptides and proteins on *n*-alkylsilicas and *n*-alkylagaroses involves multivalent interactions between hydrophobic and polar domains on the surface of the ionised solute molecules and heterogeneous binding sites on the non-polar stationary phase. Retention under regular reversed-phase chromatographic conditions is mainly due to the hydrophobic expulsion of the solute molecules from a polar hydro-organic mobile phase, *i.e.* the process is mobile phase-driven and both ΔS and ΔH are positive. At concentrations below 0.1% v/v, alcohols and other dipolar organic solvents can stabilise[39] protein structure, whereas at higher concentrations, they usually induce[40–44] either reversible deformation or denaturation. The deforming effects of most organic solvents can be attributed to regional disruption of the hydrophobic interactions between the non-polar side-chains in the protein by prefer-

ential solvation processes. A considerable proportion of the surface of a typical native globular protein is hydrophobic in so far that the atoms in these domains are not able to form hydrogen bonds. Addition of an organic solvent modifier to an aqueous medium containing a native polypeptide or protein will, in general, alter the hydration structure of the polypeptide or protein and this results in a change in thermodynamic equilibrium of the folded native structure. Denaturation or partial unfolding may follow, depending on the structure of the polypeptide or protein. In either case, there will be an increase in the surface contact between the protein and the solvent. If the system also contains a solid surface less polar than the hydro-organic solvent combination, preferential expulsion of the partially or fully unfolded protein from the liquid to the stationary phase will occur, where further structural deformation or secondary adsorption effects may transpire.

Although studies on kinetics of protein adsorption and desorption at non-polar stationary phase surfaces such as alkylsilicas potentially offer much more useful information than thermodynamics on the mechanism of protein retention, accumulation and analysis of kinetic data is more complex. One way kinetic information obtained from chromatographic investigations can be appraised and the relative importance of solute conformational effects and other secondary retention effects can be determined is through an analysis of band-broadening effects. A paradox may arise, however, with large polypeptides and proteins in the sense that elution conditions which eliminate (or greatly reduce) secondary retention effects may also simultaneously induce solute unfolding in either the mobile phase or the stationary phase (or both). For the purposes of the present investigation we chose a primary buffer combination of 30 m*M* sodium dihydrogen phosphate and 15 m*M* orthophosphoric acid, pH 2.1, and retentions were adjusted over appropriate k' values with various percentages of acetonitrile. Similar phosphate-based elution conditions have been widely used in the past[1,2,10,29], particular under linear gradient elution conditions, where good bandshape and medium-to-high solute recoveries have been observed. The significant band dispersion observed in the present investigation with the higher-molecular-weight polypeptides and proteins chromatographed under isocratic conditions was thus not completely anticipated. In fact, the data suggest that the interaction of native (or reversibly unfolded) polypeptides and proteins with alkylsilicas is generally much more complex than implied by the two-state equilibrium model described by eqn. 1. In this context, it is worth contrasting the efficiency data for the small peptides, *e.g.* angiotensin I, where the H_{eff} value on the 7.3-nm octadecylsilica packing increased to *ca.* 750 μm at a k' value of 9.5 and the efficiency data for the small proteins on the same column, where *e.g.* the H_{eff} value of hen lysozyme was *ca.* 12,500 μm at a k' value of only 0.3. These values can be compared with efficiency data reported by Meek and Rosetti[45] for insulin, where on a 8-nm porosity, 5-μm particle at a flow-rate of 2 ml/min the H value was found to be *ca.* 200 μm at $k' = 1$ to 2. One explanation which would account for the large H_{eff} value differences at equivalent k' values noted in the present investigation between conformationally mobile small peptides and the more highly structured globular proteins involves the participation of (at least) a three-state retention model. The simplest expression of this process at the stationary phase surface can be depicted as

$$S + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} S\,B \underset{k_{31}}{\overset{k_{13}}{\rightleftharpoons}} S^*\,B \underset{k_{41}}{\overset{k_{14}}{\rightleftharpoons}} S^* + B \qquad (15)$$

Equivalent equations can also be written for slow–fast interconversions of the native protein with unfolded forms, induced by the mobile phase composition in the flowing eluent.

In common with the earlier two-state model, the present hypothesis also assumes that secondary retention effects due to heterogeneity of the stationary phase binding sites, traditionally attributed to silanophilic effects, do not significantly contribute to the H_{eff} measurements. Whether such an assumption is valid, and whether mobile phase compositions that correct peak tailing due to silanophilic interactions also mediate significant conformational unfolding of polypeptides and proteins clearly deserves further detailed investigations. However, recent studies[13,46,47] from several laboratories have attempted to resolve these questions. Although in its present form of eqn. 15, the three-state model may still oversimplify the overall retention mechanism for proteins separated under reversed-phase conditions, this model nevertheless appears to provide a useful approximation for a number of experimental findings. For example, according to this model, the concentration of the protein, S, in the stationary phase, B, changes biphasically with two different relaxation times, τ_1 and τ_2, depending on the chromatographic conditions. If the overall time constant for the process of equilibration between S and a solvent-ordered, unfolded structure, S*, which does not have exactly the same tertiary shape, is comparable with the time of passage of S through the column, then several significant effects may be manifested. Firstly, individual peaks or composite skewed peaks, corresponding to S and S*, may be detected in a chromatographic profile. Following recovery, the protein may be distributed in fractions containing the native and denatured forms with different retention times as seen for soybean trypsin inhibitor[46], papain[47], ribosomal proteins[48] and hormonal proteins[13,49]. Secondly, in chromatographic separations involving long dwell and/or elution times and low elution velocities the mass (and biological) recovery of S will be low when when $(k_{12} + k_{21}) \ll k_{31} + k_{13}/(1 + k_{21}/k_{12})$. In these circumstances some molecules of S will be statistically interconverting to S* throughout the overall passage through the column, *i.e.* the concentration of S will change monotonicly. Since S* is a partially or completely unfolded form of S, in this state it will behave in reversed-phase HPLC as a more hydrophobic substance than native S. As a consequence, the native form, S, will have a shorter retention time than the unfolded form, S*, under reversed-phase HPLC conditions with alkylsilicas. Depending on the extent of unfolding, selectivity reversals may become evident when data for different proteins, separated with aqueous–organic solvent combinations on alkylsilica, are compared with data for the same proteins, separated under high salt hydrophobic interaction chromatographic conditions as, for example, has been reported[50] for ovalbumin and lysozyme. Furthermore in its unfolded state, S*, the protein may become partially or completely entrapped at the stationary phase surface owing to changes in its hydrodynamic radius and in the nature of the multivalent binding. Such behaviour would account for the phenomenon of 'ghosting', commonly observed[1,2,7,10,46,51] in the gradient elution of hydrophobic proteins on alkylsilicas. Thirdly, the choice of column dimensions will have

a significant bearing on whether native S can be recovered. Although in general terms column length does not appear to be an important factor in the retention and resolution of polypeptides with alkylsilicas in analytical separations under gradient conditions, column length does appear to influence recoveries in semipreparative separations, as expected on the basis of these relaxation time arguments. Fourthly, changes in the accessible surface area, ligand density, *n*-alkyl chain length and end-capping procedures will all influence these relaxation processes and thus potentially result in anomalies in chromatographic performance and recovery as shown, for example, by ovalbumin[10,29]. Finally, solvent composition will affect the various relaxation rates by which the solute molecules rearrange themselves on the stationary phase surface. The "all-or-nothing" recovery of some polypeptides or proteins, such as elastase or CNBr core fragments of thyroglobulin[52], when either water–acetonitrile or water–propanol mobile phases of similar relative elution strengths are employed, may be one manifestation of these effects. Experiments currently under investigation should clarify further these processes which lead to protein deformation at stationary phase surfaces.

SYMBOLS

A	Knox parameter in eqn. 7; due to eddy diffusion and slow mass transfer across the moving liquid film
a_s	Peak asymmetry factor
b	Gradient steepness parameter
B	Knox parameter in eqn. 7; due to longitudinal diffusion
C	Knox parameter in eqn. 7; due to mass tranfer in the stationary phase
D_i	Intraparticulate diffusion coefficient
D_m	Diffusion coefficient in the mobile phase
d_p	Particle diameter
D_s	Diffusion coefficient in the stationary phase
D_{sz}	Diffusion coefficient in the stationary zone
G	Band compression factor in gradient elution
h_{disp}	Reduced plate height term for longitudinal molecular diffusion and eddy dispersion
$h_{c,diff}$	Reduced plate height term for resistance to mass transfer at the particle boundary
h_{eff}	Effective reduced plate height
H_{eff}	Height equivalent of a theoretical plate as derived from peak moments
$h_{i,diff}$	Reduced plate height term for intraparticulate diffusion to mass transfer in stagnant mobile-phase pools
h_{kin}	Reduced plate height term for kinetic resistance for solute binding
H_{obs}	Height equivalent of a theoretical plate, as calculated from t_a and w_h
K	Thermodynamic equilibrium constant
k'	Capacity factor or mass distribution ratio
k''	Zone capacity factor, as defined in eqn. 9
k_a	Rate constant for association in eqn. 1
k_d	Rate constant for dissociation in eqn. 1
$k'_{i,w}$	Capacity factor of solute, i, in neat water
k_o	Ratio of intraparticulate void volume to the interstitial void volume explored by the solute molecule

k_{12},k_{21},k_{13} k_{31},k_{14},k_{41}	Rate constants for association, dissociation and unfolding at the stationary-phase surface in eqn. 15
L	Column length
n	Effective peak number
N_{eff}	Theoretical plate number, as derived from peak moments
N_{obs}	Theoretical plate number, as calculated from t_a and w_h
S	Solvent strength parameter for solute
t_a	Retention time at apex of peak
t_m	Retention time at centre of gravity of peak
t_o	Retention time of a non-sorbed solute
t_w	Dwell time the solute spends at the head of the column
t_z	Residence time in stationary zone
u_e	Linear interstitial mobile-phase velocity
u_o	Linear velocity of mobile zone
$V_{i,o}$	Interstitial void volume
V_o	Void volume
$V_{p,o}$	Intraparticulate void volume
w_h	Peak width at half height
β	Fraction of mobile phase that is stagnant
γ	Obstruction factor of the column packing in eqn. 13
ε_e	Interstitial porosity
ε_i	Intraparticulate porosity
ν	Reduced interstitial velocity of the mobile phase
ν_o	Reduced velocity of the mobile zone
σ_G	Standard deviation of a Gaussian constituent of a skewed peak
σ_t^2	Peak variance in units of time
τ_s	Time constant of the exponential modifier of a skewed peak
τ_1, τ_2	Relaxation times for the biphasic process, described by eqn. 15
φ	Phase ratio
ψ	Volume fraction of the organic solvent modifier

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