Journal of Chromatography, 593 (1992) 103–117
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2466

High-performance liquid chromatography of amino acids, peptides and proteins

CXV☆. Thermodynamic behaviour of peptides in reversed-phase chromatography

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ABSTRACT

The thermodynamic behaviour of three peptides, bombesin, β-endorphin and glucagon, was studied under reversed-phase high-performance liquid chromatographic conditions. Experimental data related to the interactive surface contact area (S values) and solute affinity ($\log k_0$) were derived over a range of temperatures between 5 and 85°C. These experimental conditions allowed changes in the secondary structure of the solute to be monitored. The influence of the nature of the stationary phase ligand on the relative conformational stability of the three peptides was analysed by acquiring data with *n*-octadecyl silica (C_{18}) and *n*-butyl silica (C_4) sorbents. Values for the relative changes in entropy and enthalpy associated with the interactive process were also determined. The results provide further insight into the factors involved with the stabilization of secondary structure and the mechanism of the interaction of peptides with hydrophobic surfaces.

INTRODUCTION

The development of interactive modes of chromatography to investigate the physico-chemical nature of peptide and protein surface interactions has advanced considerably over the past decade. These powerful techniques have gained wide recognition as rapid and extremely useful procedures for elucidating, in structural and molecular terms, biopolymer behaviour at liquid–solid interfaces under a wide range of experimental conditions [2–4]. The hydrophobic modes of chromatography, namely reversed-phase high-performance liquid chromatography (RP-HPLC) and hydrophobic interaction chromatography (HIC), have especially been used in strategies employed for studies of the mechanistic basis of chromatographic separations. The usefulness of modern LC techniques stems from the ability of the stationary phase ligands to probe the solute surface. Different ligands may be employed to explore different physical and chemical properties of the interfacial region of the solute recognized by the stationary phase. The fact that this approach, aptly coined chromato-topography [5,6], also enables kinetic properties of the solute to be followed thus allows insight into the dynamic nature of the protein–ligand interaction. This attribute of modern LC methods with appropriate on-line detection systems clearly represents an advantage over many of the conventional techniques such as X-ray crystallography and other static methods of spectroscopic measurement hitherto used to evaluate biopolymer–ligand interactions.

It was postulated 15 years ago [7] that the hydrophobic surface in RP-HPLC may be a useful probe for investigating amphipathic helices and

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0021-9673/92/$05.00

other helices of biopolymers induced or stabilized in hydrophobic environments and the behaviour of peptides and proteins at a hydrophobic interface, such as a lipid bilayer. This paper examines the potential of this technique and the validity of the above considerations by examining the thermodynamic behaviour of three biologically significant peptides, bombesin, β-endorphin and glucagon. The secondary and tertiary structures of these peptides in solution have been characterized [8–13], and each contains significant amounts of α-helical structure under certain conditions. The relative stability of the secondary structure components within these peptides was studied by varying the temperature and the stationary phase composition upon which they were allowed to interact in the RP-HPLC mode.

EXPERIMENTAL

Apparatus

All chromatographic measurements were performed on a Perkin-Elmer (PE) Series 4 chromatograph (Perkin-Elmer, Norwalk, CT, USA) utilizing a PE ISS-100 autosampler, PE LC-95 UV–visible spectrophotometer and a PE 7500 professional computer with CHROM 3 software. All peak profiles were routinely monitored at 215 nm and stored on the Winchester disk of the PE 7500 and processed simultaneously by a PE LCI-100 computing integrator. Further peak analysis was performed using software packages included in the CHROM 3 framework. Temperature was controlled by either immersing the column in a thermostated column water-jacket coupled to a recirculating cooler (FTS Systems, New York, USA) or by an ICI TC 1900 column oven (ICI Instruments, Dingley, Victoria, Australia).

Chromatography was performed on Bakerbond wide-pore butylsilica and octadecylsilica columns (J. T. Baker, Phillipsburg, NJ, USA) with dimensions of 250 × 4.6 mm I.D. and containing sorbents of 5 μm nominal particle size and 30 nm average pore size.

All pH measurements were made with an Orion (Cambridge, MA, USA) Model SA520 pH meter.

Chemicals and reagents

Acetonitrile (HPLC grade) was obtained from Mallinckrodt (Paris, KY, USA) and trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA). Water was distilled and deionized in a Milli-Q system (Millipore, Bedford, MA, USA). N-Acetyl-L-tryptophanamide, N-acetyl-L-phenylalanine ethyl ester, penta-L-phenylalanine, bombesin and glucagon were all obtained from Sigma (St. Louis, MO, USA) and were of >95% purity by RP-HPLC and amino acid composition analysis. β-Endorphin (of purity >95%) was obtained from Organon (Oss, Netherlands) and Sigma; both batches gave identical HPLC profiles.

Chromatographic procedures

Bulk solvents were filtered and degassed by sparging with nitrogen. Linear gradient elution was performed using 0.1% TFA in water (buffer A) and 0.09% TFA in acetonitrile–water (65:35) (buffer B) over gradient times of 30, 45, 60 and 90 min with a flow-rate of 1 ml/min at temperatures of 5, 15, 25, 37, 45, 55, 65, 75 and 85°C. Peptide solutions were prepared by dissolving the solute at a concentration of 0.5 mg/ml in 0.1% TFA and the injection size varied between 1 to 5 μg. All data points are derived from at least duplicate measurements with retention times between replicates varying typically by less than 1%.

The column dead volume was taken as the retention time of the non-interactive solute, sodium nitrate. Various chromatographic parameters were calculated using the Pek-n-ese program [14] written in Pascal for an IBM PC, and statistical analysis involved ANOVA linear regression analysis. In all figures presented, the standard deviations of replicate experiments were as shown, or smaller than the data points.

RESULTS AND DISCUSSION

Theoretical background

The retention of a solute in interactive modes of chromatography is dictated by the equilibria established for the distribution of the solute in the mobile and stationary phases as described by

$$K = \frac{[P]_s}{[P]_m} \quad (1)$$

where $[P]_s$ and $[P]_m$ are the concentrations of solute in the stationary and mobile phases respectively. The dependence of the capacity factor, k', on the

chromatographic equilibrium constant, K, can be equated to

$$k' = \phi K \quad (2)$$

where ϕ is the phase ratio, that is, the ratio of volumes of stationary to mobile phases in the chromatographic bed.

For linear gradient elution systems, a mathematical model known as the linear solvent strength (LSS) model has been shown to provide useful information associated with the physico-chemical properties of the solute [3,14,15].

Under regular reversed-phase gradient elution conditions, a linear relationship typically exists between the medium capacity factor, $\log \bar{k}$, and the median organic mole fraction, $\bar{\psi}$, according to the empirical equation.

$$\log \bar{k} = \log k_0 - S\bar{\psi} \quad (3)$$

The values of S and $\log k_0$ can then be determined by multivariate linear regression analysis of $\log \bar{k}$ *vs.* $\bar{\psi}$ plots according to eqn. 3.

The significance of the slope, S, of $\log \bar{k}$ *vs.* $\bar{\psi}$ plots in RP-HPLC resides in its relationship to the magnitude of the surface contact area and the number of interactive sites established at the interface between the solute and the stationary phase ligands. The S value as evaluated through eqn. 3 can be related to the hydrophobic contact area as derived from the solvophobic theory [7,16] as follows:

$$\log k' = \log k_0 - \gamma \cdot \frac{N\Delta A_h + 4.836N^{\frac{1}{3}}(\kappa^e - 1)V^{\frac{2}{3}}}{2.303RT} \quad (4)$$

where ΔA_h is the relative hydrophobic contact area, γ is the mobile phase surface tension, which is directly related to the organic mole fraction, $\bar{\psi}$, N is Avogadro's number, V is the mean molar volume of the solvent, R is the gas constant and T is the absolute temperature. The parameter κ is defined as the ratio of the energy required for the formation of a cavity with the surface area equal to solute surface area and the energy required to extend the planar surface of the liquid by the same area. The value of $\log k_0$ is related to the change in free energy associated with the adsorption of the solute in the absence of organic modifier, and can thus be related to the affinity of the solute for the stationary phase at $\bar{\psi} = 0$ (*i.e.*, initial conditions).

The determination of these parameters at different temperatures allows a detailed analysis of the thermodynamic behaviour of the solute during the interaction with the stationary phase. The thermodynamic equilibrium constants can be equated to the overall standard unitary free-energy changes (ΔG^0_{assoc}) associated with the transfer of the solute from the mobile phase to the stationary phase, such that

$$\log K = -\frac{\Delta G^0_{assoc}}{RT} \quad (5)$$

Solute retention under equilibrium binding conditions can therefore be described as

$$\log \bar{k} = \frac{-\Delta H^0_{assoc}}{RT} + \frac{\Delta S^0_{assoc}}{R} + \log \phi \quad (6)$$

where ΔH^0_{assoc} and ΔS^0_{assoc} are the changes in enthalpy and entropy, respectively, for the association

TABLE I

PHYSICAL DATA FOR THE SOLUTES STUDIED

Solute	Sequence[a]	Molecular weight
N-Acetyltryptophanamide	N-Ac-W-NH_2	230
N-Acetylphenylalanine ethyl ester	N-Ac-F-OEt	210
Penta-L-phenylalanine	H_2N-FFFFF-OH	760
Bombesin	H_2N-XQRLGNQWAVGHLM-OH	1640
β-Endorphin	H_2N-YGGFMTSEKSQTPLVFKNAIIKNAYKKGE-OH	3470
Glucagon	H_2N-HSQGTFTSDYSKYLDSRRAQDFVQWLMNT-OH	3520

[a] Ac = Acetyl.

process. The ΔH^0_{assoc} and ΔS^0_{assoc} values can then be derived from the slope and intercept values determined by regression analysis of Van 't Hoff plots (*i.e.* log $\bar{k}$ *vs.* $1/T$). Linear relationships in such plots are generally obtained for small organic molecules with no significant secondary or tertiary structure. However, the interactive surface of a peptide or protein solute in RP-HPLC will consist of sequentially and non-sequentially linked amino acids brought together spatially as a result of the secondary or tertiary constraints of the solute structure. For solutes with well developed secondary structure, chromatographed over a wide range of temperatures, significant deviation from linearity has been observed in Van 't Hoff plots [3]. This behaviour is a result of temperature-induced conformational changes which affect the molecular composition of the interactive surface of the solute and will therefore be reflected in both the retention behaviour of the solute and the thermodynamic parameters derived from Van 't Hoff plots.

Dependence of S and log k_0 on temperature

The retention behaviour of a range of peptide solutes with known α-helical structure [8–13] was studied to provide further information on the factors which influence the chromatographic behaviour of peptides and proteins. The gradient elution behaviour of two amino acid derivatives, N-acetyltryptophanamide and N-acetylphenylalanine ethyl ester and four peptides, penta-L-phenylalanine and the peptide hormones bombesin, β-endorphin and glucagon, was measured under a range of experimental conditions. The molecular weights and sequences of these solutes are summarized in Table I.

As solution conformation is substantially influenced by environmental temperature, and can be generally perturbed within the temperature range available in chromatographic studies, the influence of temperature on the RP-HPLC behaviour of these peptides was studied. Gradient elution data were accumulated at gradient times between 30 and 90 min at a flow-rate of 1 ml/min and at temperatures ranging between 5 and 85°C. In order to study the influence of different hydrocarbonaceous ligands, samples were chromatographed on C_4 and C_{18} silicas. Plots of log $\bar{k}$ *vs.* $\bar{\psi}$ were used to derive S and log k_0 values for each solute and the temperature dependencies of these values are represented graphically in Figs. 1–5.

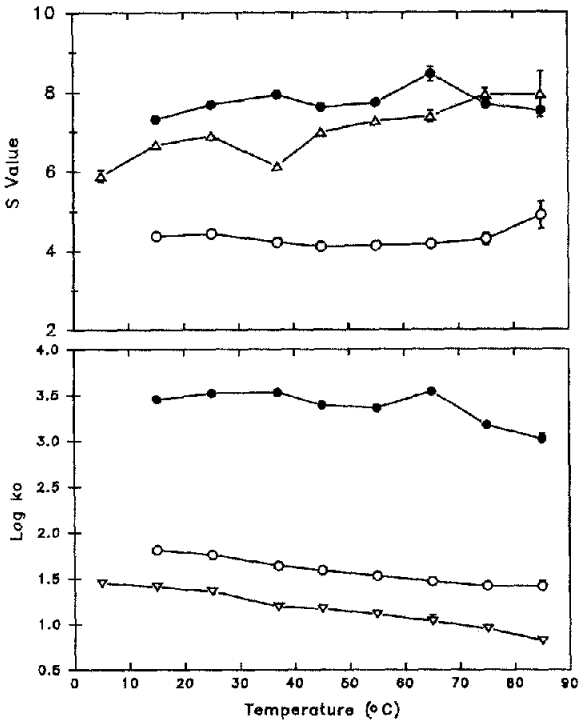

Fig. 1. Top, dependence of S values on temperature for the control solutes (●) penta-L-phenylalanine, (○) N-acetylphenylalanine ethyl ester and (△) N-acetyltryptophanamide chromatographed on the C_{18} stationary phase. Bottom, dependence of log k_0 on temperature for the same solutes chromatographed on the C_{18} stationary phase.

N-Acetyltryptophanamide, N-acetylphenylalanine ethyl ester and penta-L-phenylalanine were chromatographed as control solutes. These solutes are small molecules with no secondary structure. Changes in retention behaviour over the temperature range studied will therefore not be related to conformational changes in the interactive structure. N-Acetyltryptophanamide was only retained on the C_{18} stationary phase. These three solutes on the C_{18} stationary phase had essentially constant S values over the range of temperatures examined and the corresponding affinity (log k_0) demonstrated a constant or small uniform decrease in value with increases in temperature (Fig. 1). Similar trends in the temperature dependencies of the S and log k_0 values were also observed for these solutes when chromatographed on the C_4 phase, as depicted in Fig. 2. However, the magnitude of the affinity of the

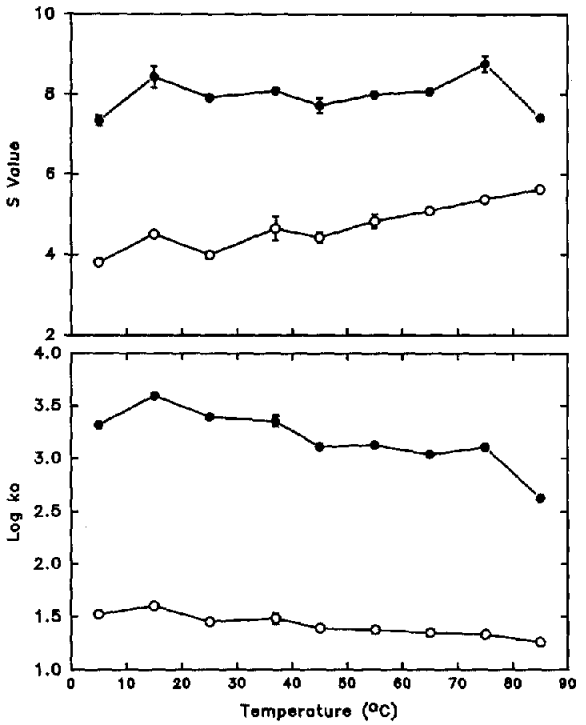

Fig. 2. Top, dependence of S values on temperature for the control solutes (●) penta-L-phenylalanine and (○) N-acetyl-phenylalanine ethyl ester chromatographed on the C_4 stationary phase. N-Acetyltryptophanamide was not retained at high temperatures on this phase. Bottom, dependence of log k_0 on temperature for the same solutes chromatographed on the C_4 stationary phase.

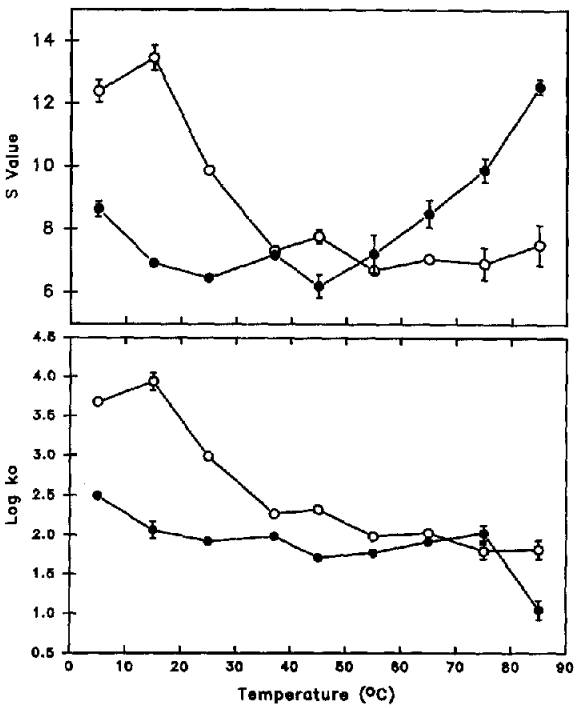

Fig. 3. Top, dependence of S values on temperature for bombesin chromatographed on the (○) C_{18} and (●) C_4 stationary phases. Bottom, dependence of log k_0 on temperature for bombesin on these two stationary phases.

control solutes chromatographed on the C_{18} stationary phase was generally higher than that on the C_4 stationary phase. Thus, for these solutes chromatographed over a wide range of temperatures, the two stationary phases behaved as would be empirically predicted on the basis of the relative hydrophobicity of the ligands when conformationally rigid solutes are chromatographed. It would also appear that similar interactive regions are involved in the interaction between the control solutes and the two stationary phase types, which is reflected in the similar magnitude for the derived chromatographic S-values for both the C_{18} and C_4 phases.

As these control solutes are small organic molecules, the results in Figs. 1 and 2 represent the degree of change in S and log k_0 which would be expected for solutes which undergo no structural perturbation with increased temperature. Penta-L-phenylalanine displays some minor fluctuations with temperature, as would be expected for a molecule of greater conformational flexibility. However, similar S values were obtained with both C_4 and C_{18} ligands. Moreover, these solutes represent useful probes for the changes in *n*-alkyl ligand structure which may occur at different temperatures.

Fig. 3 illustrates the relationship between S values and temperature for bombesin on the two different stationary phases studied. A transition at *ca.* 25°C corresponding to two different interactive structures with high and low contact areas was observed on the C_{18} phase. The affinity *vs.* temperature plot (Fig. 3) also indicates a similar transition with significantly elevated log k_0 values in the 5–25°C range for bombesin on the C_{18} phase. Thus, at higher temperature, bombesin is interacting through a much smaller proportion of its molecular surface area and with a diminished affinity.

In contrast to the C_{18} phase, bombesin shows

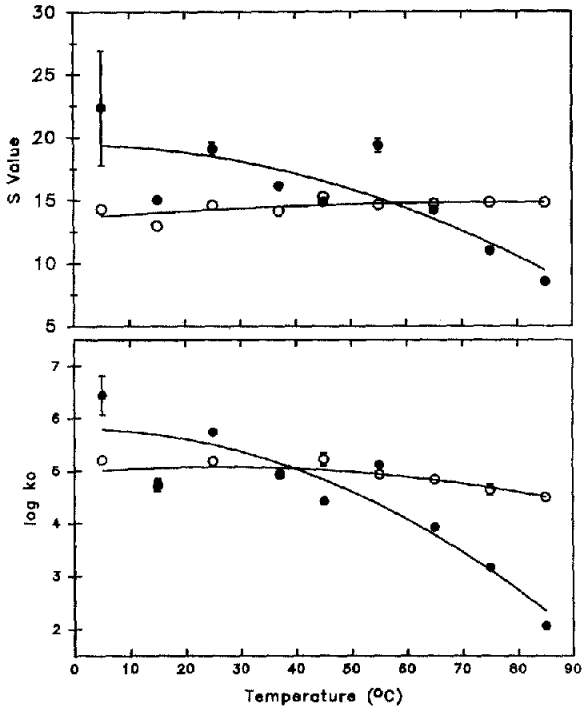

Fig. 4. Top, dependence of S values on temperature for β-endorphin chromatographed on the (○) C_{18} and (●) C_4 stationary phases. Bottom, dependence of log k_0 on temperature for β-endorphin on these two stationary phases.

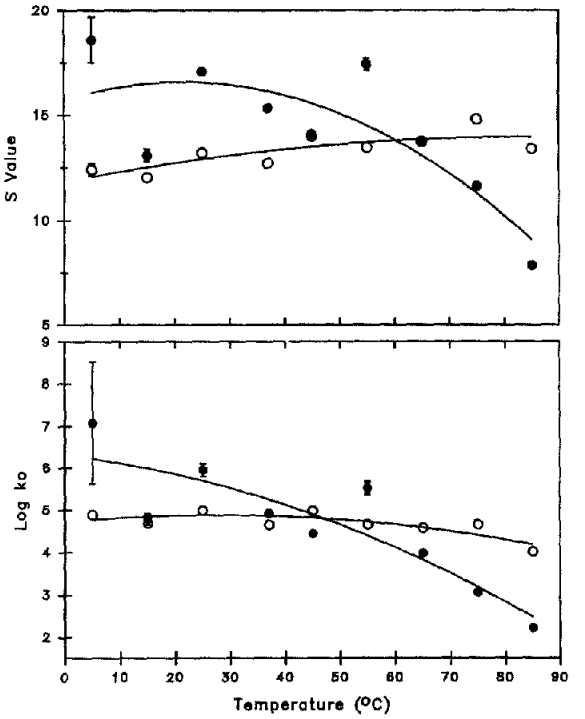

Fig. 5. Top, dependence of S value on temperature for glucagon chromatographed on the (○) C_{18} and (●) C_4 stationary phases. Bottom, dependence of log k_0 on temperature for glucagon on these two stationary phases.

marked increases in S value on the C_4 phase at temperatures above 50°C, which coincided with a decrease in affinity for this region of temperature. This result implies that whilst a larger surface area is exposed by bombesin to the C_4 ligands at these higher temperatures, the affinity of the peptide for the sorbent at high temperatures is lower than when the solute exists with a more compact interactive structure at the lower temperature range. These observations suggest that at higher temperatures bombesin presents a more diffuse surface distribution of hydrophobic amino acids to the probing C_4 stationary phase ligands.

The relative changes in affinity and chromatographic contact area with temperature for bombesin are much greater compared with the control solutes. In particular, on the C_{18} phase, the S value for bombesin changes from 12.5 to 7.0 at *ca.* 25°C, whereas the S value for penta-L-phenylalanine remained constant. The results for these two solutes chromatographed on the C_4 phase indicate an opposite trend, where at temperatures below 50°C similar S values are observed both for penta-L-phenylalanine and bombesin, but at the higher temperatures significantly higher S values and hence contact areas are observed for bombesin. Penta-L-phenylalanine also had a relatively constant S-value over the temperature range investigated on the C_4 phase.

The affinity values also reflect these differences in chromatographic behaviour between penta-L-phenylalanine and bombesin. On the C_{18} phase, bombesin has a lower affinity compared with the control solute at high temperatures, whereas similar values for both solutes are observed at the low temperatures. Penta-L-phenylalanine demonstrated a small but uniform decrease in affinity with increasing temperature, whereas transitions were apparent for bombesin. Comparison of log k_0 values determined for both solutes on the C_4 phase indicate that bombesin has overall lower affinity values than the control solute over the temperature range stud-

ied, but both solutes demonstrated a uniform decrease in affinity with temperature. These significant changes in chromatographic behaviour of bombesin are consistent with changes in the interactive structure of bombesin relative to penta-L-phenylalanine and the other control solutes. Hence it appears that bombesin adopts, in the presence of *n*-alkyl ligands, some degree of secondary structure, which may be helical as bombesin is known to possess an α-helical structure in hydrophobic conditions [8,9].

The differences in the behaviour of bombesin on the C_4 and C_{18} stationary phases also indicates that the two structurally disparate ligands are either probing different areas of the solute surface or that bombesin adopts a different structure in the presence of the two ligands. The difference in dynamic behaviour of the two stationary phases is well documented [17,18], with the C_4 ligands tending to be more sterically rigid than the C_{18} ligands, which have the potential to self-associate owing to their higher degree of flexibility. The C_4 stationary phase has been described as a "picket-fence" with C_4 ligands projecting away from the silica surface [19] into the mobile phase whereas the C_{18} stationary phase comprises a "lawn" of alkyl chains forming a hydrophobic surface that coats the silica particles. Other workers have shown that transitions occur in the structure of longer chain alkyl ligands. These phase transitions have been shown to cause non-linear relationships between solute retention and temperature. Gilpin and Squires [20] studied phase transitions for C_8-, C_9- and C_{10}-alkylsilicas and determined phase transition temperatures of *ca.* 40, 50 and 60°C, respectively. The temperature increment of 10°C in the phase transition with each additional methylene group added suggests that the theoretical transition for the C_{18} stationary phase would be well outside the experimental temperature range utilized in this study, and that changes in the ligand structure do not account for the observed changes in retention behaviour of bombesin.

Jinno *et al.* [21] recently conducted a study on a series of polymeric C_{18} stationary phases and observed a non-linear retention dependence on temperature for planar polycyclic aromatic hydrocarbon solutes for some of the stationary phases investigated. This retention behaviour was attributed to changes in the ligand structure between 40 and 50°C. Although this transition range is not in accordance with the work of Gilpin and Squires, different solvent systems were employed. Jinno *et al.* [21] used an organic solvent system of methanol–dichloromethane (80:20, v/v), whereas Gilpin and Squires used a totally aqueous system.

Although it is possible that a phase transition may occur in the experimental temperature region used in this study, several observations suggest that this phenomenon does not significantly influence the retention behaviour of the solutes employed in this study. First, a linear dependence of the derived retention parameters on temperature was obtained for the control solutes, which strongly suggests that should changes occur in the structure of the stationary phase ligand, they are not responsible for the observed peptide transitions. Additionally, of the peptides studied only bombesin shows a significant transition in S value on the C_{18} stationary phase. Hence, if a significant change occurred in the structure of the ligand, it should be manifested in the retention properties of all solutes. These observations strongly suggest that the observed non-linear temperature dependence of the derived retention parameters is due to solute-specific structural changes and not phase transitions of the immobilized ligands.

The dependence of S and $\log k_0$ on temperature for the two larger peptides, β-endorphin and glucagon, on the C_{18} phase are shown in Figs. 4 and 5, respectively. The behaviour observed on the C_{18} phase for both β-endorphin and glucagon indicates a gradual increase in chromatographic S value with increasing temperature. The magnitude of this change was smaller than that observed for bombesin (Fig. 3) with the same phase. The plots of $\log k_0$ *vs.* temperature indicate a small, uniform decrease in affinity between β-endorphin or glucagon and the C_{18} phase over the temperature range examined.

For both β-endorphin and glucagon on the C_4 stationary phase, a sharp decrease in S values between 50 and 85°C was observed (Figs. 4 and 5 for β-endorphin and glucagon, respectively), as opposed to bombesin, which underwent a rapid increase in S value over this temperature range. This decrease in chromatographic contact area for both of these solutes also corresponded to a large decrease in affinity (Figs. 4 and 5), which is consistent with a decrease in the number of amino acid residues participating in the interaction at these higher

TABLE II

GIBB'S FREE ENERGY CHANGES FOR THE SOLUTES STUDIED ON BOTH PHASES

Temperature (°C)	Change in Gibb's free energy (ΔG^0_{assoc}) (kJ mol^{-1})					
	Bombesin		β-Endorphin		Glucagon	
	C_{18}	C_4	C_{18}	C_4	C_{18}	C_4
5	−9.4	−6.6	−12.9	−17.2	−12.2	−15.7
15	−10.3	−5.8	−12.2	−12.3	−12.1	−12.3
25	−8.3	−5.7	−13.8	−15.6	−13.3	−15.1
37	−6.8	−6.1	−13.7	−13.6	−12.9	−13.7
45	−7.1	−5.5	−14.8	−12.7	−14.1	−12.7
55	−6.4	−5.8	−14.5	−16.0	−13.7	−15.0
65	−6.7	−6.4	−14.6	−12.2	−13.9	−12.1
75	−6.3	−6.9	−14.5	−9.9	−14.5	−10.2
85	−6.5	−4.2	−14.5	−7.7	−13.1	−7.3

TABLE III

CHANGES IN ENTHALPY AND ENTROPY FOR THE SOLUTES STUDIED

Solute	$\bar{\psi}$	C_{18}-silica[a]		C_4-silica[a]	
		ΔH^0_{assoc} (kJ mol^{-1})	ΔS^0_{assoc} (J mol^{-1} K^{-1})	ΔH^0_{assoc} (kJ mol^{-1})	ΔS^0_{assoc} (J mol^{-1} K^{-1})
N-Acetylphenylalanine ethyl ester	0.1	−5.4	−5.9	−4.6	−5.3
	0.3	−6.0	−14.7	−8.4	−25.3
N-Acetyltryptophanamide[b]	0.1	−8.2	−20.7		
	0.3	−12.2	−45.0		
Penta-L-phenylalanine	0.1	−5.8	−7.7	−8.6	−5.0
	0.3	−5.1	7.3	−7.9	−17.0
Bombesin	0.1	9.8^{5-15}	58.6		
		-36.3^{15-37}	−102.1	−5.6	−4.5
		-11.0^{45-85}	−19.1		
	0.3	1.0^{5-55}	6.2	-1.8^{4-45}	−5.2
		-16.8^{65-85}	−47.5	-29.9^{45-85}	−94.4
β-Endorphin	0.1	-0.8^{5-45}	30.7	-15.6^{25-55}	−18.3
		-15.8^{45-85}	−16.0	-69.4^{55-85}	−180.0
	0.3	-5.0^{5-45}	−7.3	-5.9^{5-45}	−15.2
		-14.4^{45-85}	−36.6	-27.2^{45-85}	−82.4
				-17.8^{55-85}	−4.8
Glucagon	0.1	-1.9^{5-45}	26.1	-39.9^{25-45}	−97.6
		-17.0^{45-85}	−23.7	-65.6^{55-85}	−169.0
	0.3	-7.2^{5-45}	−13.0	-1.7^{15-25}	2.1
		-18.2^{45-85}	−47.4	-15.9^{25-75}	−45.4
				4.3^{75-85}	12.6

[a] Superscripts denote the temperature region (°C) of the Van 't Hoff plot used to derive the corresponding parameters.
[b] N-Acetyltryptophanamide was not fully retained over the temperature studied on the C_4 stationary phase.

temperatures. As the compositional properties of the interactive region will determine both the affinity and the magnitude of the contact area for a particular solute, the results of this study suggest that the changes in retention behaviour with increasing temperature for these three peptidic solutes are due primarily to the conformational changes. These conformational changes over the temperature range investigated will in turn dictate the accessible interactive surface that will be presented to the probing stationary phase ligands.

Dependence of thermodynamic parameters on temperature

Consideration of the thermodynamic parameters underlying the biomolecular structural characteristics [22] of peptide and protein–surface interactions provides further insight into the chromatographic process. Changes in ΔG^0_{assoc} were calculated at the individual temperatures from log k_0 values according to eqn. 5. As adsorption is a favourable process, the derived ΔG^0_{assoc} values which are given in Table II are all negative. Van 't Hoff plots (log $\bar{k}$ *vs.* $1/T$) were also examined in order to derive the changes in enthalpy (ΔH^0_{assoc}) and entropy (ΔS^0_{assoc}) for the association process by regression analysis of the dependence of log $\bar{k}$ on $1/T$ (as described by eqn. 6). In order to assess the influence of the organic modifier concentration on the structure of the solute and on the interactive process, log $\bar{k}$ values extrapolated from two different regions of the retention plots (log $\bar{k}$ *vs.* $\bar{\psi}$) were examined. Log $\bar{k}$ values extrapolated to $\bar{\psi} = 0.1$ and 0.3 were determined and the corresponding ΔS^0_{assoc} and ΔH^0_{assoc} values are given in Table III.

Van 't Hoff plots for bombesin, β-endorphin and glucagon are shown in Figs. 6–8. Non-linear relationships were found to exist for the three large

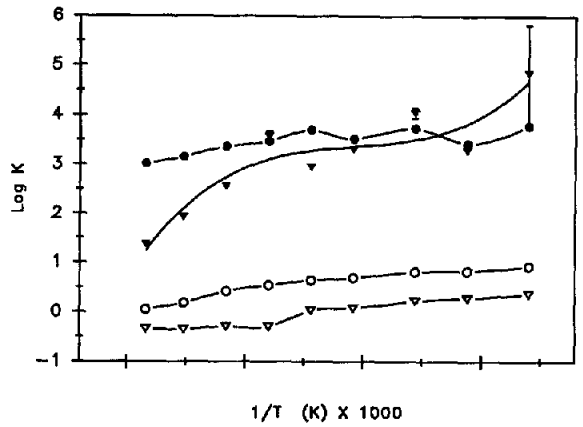

Fig. 7. Van 't Hoff plot for β-endorphin chromatographed on the (▽,▼) C_4 and (○,●) C_{18} stationary phases at the two log $\bar{k}$ values ($\bar{\psi}$ = 0.1 and 0.3, closed and open symbols, respectively).

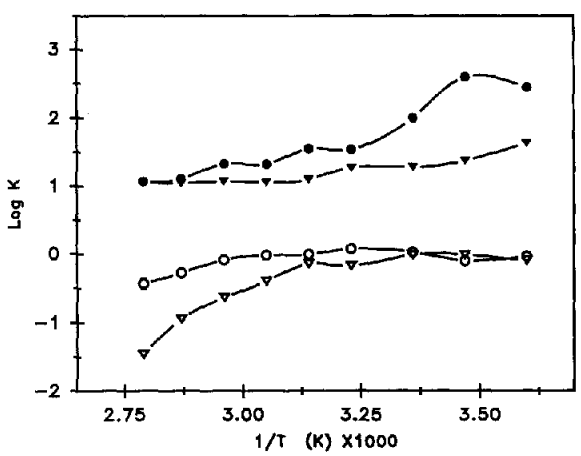

Fig. 6. Van 't Hoff plot for bombesin chromatographed on the (▽,▼) C_4 and (○,●) C_{18} stationary phases at the different log $\bar{k}$ values ($\bar{\psi}$ = 0.1 and 0.3, closed and open symbols, respectively).

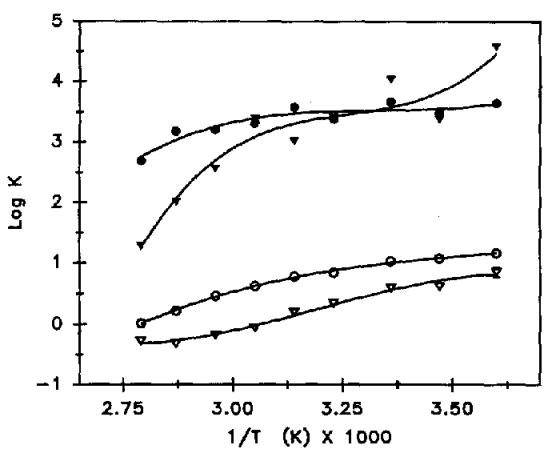

Fig. 8. Van 't Hoff plot for glucagon chromatographed on the (▽,▼) C_4 and (○,●) C_{18} stationary phases at the two log $\bar{k}$ values ($\bar{\psi}$ = 0.1 and 0.3, closed nd open symbols, respectively).

peptidic solutes. This result is in contrast to the Van 't Hoff plots for the control solutes, which generally gave linear relationships for both stationary phases examined (data not shown). The non-linear behaviour of the larger polypeptides correlates with changes observed for these solutes in retention parameters discussed in the previous section, and also provides further support for the existence of conformational changes that occur during chromatographic migration.

Two important generalizations about the entropy of the interactive process can be made [3], namely that positive values for the change in entropy for the association process (ΔS^0_{assoc}) indicate an increase in the total disorder of the system during adsorption, whereas it is inherent in the corollary that negative values of ΔS^0_{assoc} indicate an increased ordering of the system during adsorption. For the control solutes N-acetylphenylalanine ethyl ester and N-acetyltryptophanamide, negative values for ΔS^0_{assoc} were obtained for both stationary phases and at both extrapolated values of log $\bar{k}$. This result can be interpreted as an overall ordering of the system on interaction with the stationary phase, reflecting a solute that is less flexible when bound to the stationary phase than when it is in the mobile phase. In addition, values of ΔS^0_{assoc} for log $\bar{k}$ at $\bar{\psi} = 0.1$ are less negative than those at $\bar{\psi} = 0.3$. This result indicates that the solutes are more flexible in mobile phases containing organic solvents than in more aqueous solutions, which in turn suggests that they interact more readily with the organic solvent molecules than with the water molecules. This conclusion forms the basis of the solvophobic mechanism underlying retention in RP-HPLC and is also consistent with the observation that at $\bar{\psi} = 0.3$, lower log $\bar{k}$ values are obtained. As a result of this behaviour, the solute will be less constrained and the total system will be more disordered, thus accounting for the more negative ΔS^0_{assoc} values at log $\bar{k}$ with $\bar{\psi} = 0.3$.

The same trends are evident for penta-L-phenylalanine, where negative ΔS^0_{assoc} values were observed for all conditions except on the C_{18} phase at log $\bar{k}$ ($\bar{\psi} = 0.3$). A small positive ΔS^0_{assoc} value was observed under the latter conditions, indicating a possible disordering on binding to this stationary phase at the high organic solvent concentration. This result reflects a more rigid solution structure in comparison with the bound molecule, suggesting that some form of conformational change on association with the stationary phase may occur.

Bombesin on C_{18} phase. Fig. 6 displays the Van 't Hoff plots for bombesin chromatographed on both stationary phases at the two extrapolated values of log $\bar{k}$. Changes in enthalpy and entropy for the interactive process are given in Table III. Negative ΔH^0_{assoc} values were generally observed for all solutes and indicate that heat is liberated on adsorption of the solute on the stationary phase. These decreases in enthalpy are presumably related to the changes in solvation of the solute and the corresponding changes in solvation energies for the melted structure.

Two opposing processes may contribute to a change in disorder or entropy on denaturation of the solute. First, the perturbation of secondary structure requires increased solvation of the newly exposed atoms which originally formed the "internal" core. These atoms, previously involved in intramolecular interactions, would not have been solvated prior to the thermal disruption of the solute. This solvation would in turn cause a corresponding decrease in entropy of the desorbed state, owing to the decreased number of free solvent molecules. However, this process will be compensated for by an increased disordering of the unbound system due to the increase in the conformational repertoire of the more flexible peptide. Thus, on interaction with the stationary phase, increased ordering would be apparent owing to the more static nature of the bound peptide and the conformational restraints conferred by the interactive process. This interactive process would then be expected to yield overall negative ΔS^0_{assoc} values and is represented schematically in Fig. 9.

Analysis of the Van 't Hoff plot for bombesin at $\bar{\psi} = 0.1$ chromatographed on the C_{18} phase reveals a curvilinear dependence corresponding to three apparently linear temperature ranges, 5–15, 15–37 and 45–85°C, with a transition around 25°C. This transition correlates with the observed decrease in contact area noted earlier. At low temperatures, ΔS^0_{assoc} was found to equal 58.6 J mol^{-1} K^{-1}, which then decreased significantly to -102.1 J mol^{-1} K^{-1} over the transition range. At high temperatures a value of -19.1 J mol^{-1} K^{-1} for ΔS^0_{assoc} was obtained. These ΔS^0_{assoc} changes reflect an initially

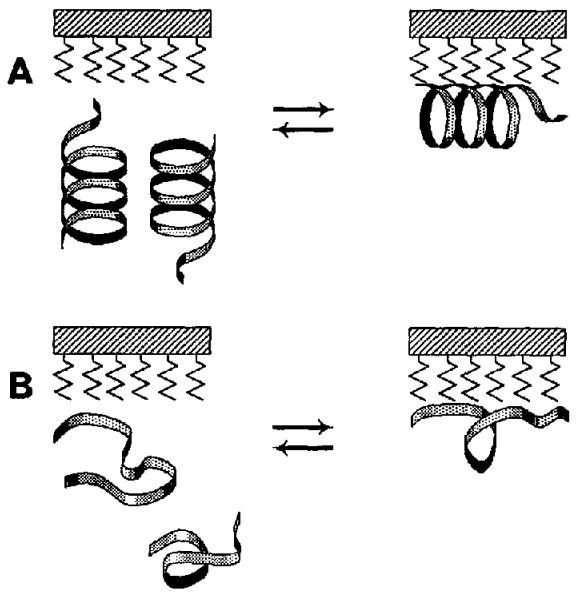

Fig. 9. Schematic view of the thermodynamic considerations involved in the interctive process of a polypeptide, with and without a well developed secondary structre, interacting with a hydrocarbonaceos ligand. In (A), the solute molecule has a rigid solution structure, which on interaction with the stationary phase undergoes conformational changes that cause disordering of the solute molecular population, resulting in a small positive value for the change in entropy ($\Delta S^0_{assoc.}$). Alternatively, the rigid solution structure may become slightly more constrained, thus yielding a small negative value of $\Delta S^0_{assoc.}$ In (B), polypeptide of random solution structure binds to the stationary phase. This induces a more constrained conformation on the solute molecular population, which in turn causes an increase in ordering of the system, leading to a large negative value of $\Delta S^0_{assoc.}$

rigid solution structure that becomes more disordered on interaction with the stationary phase at low temperatures (5–15°C). The difference between the entropy of the bound and unbound states is much larger at the transition temperature (15–37°C) than at the lower (<15°C) and higher temperatures (>37°C). These results are consistent with the peptide existing as a much more flexible molecule in the transition range, and remaining flexible at the higher temperatures. It is interesting that the 45–85°C region yielded a smaller negative ΔS^0_{assoc} than the transitional region, indicating that the molecule is still more flexible in solution than when bound, but at these temperatures either the conformational repertoire is restricted to a smaller population or the elevated temperatures cause a more rapidly "time-averaged" structure to be observed. Here the difference in the entropy of the bound structure and solution structure would not be large owing to the rapid conformational interconversions occurring to both the sorbed and desorbed states of the molecule at these temperatures.

The Van 't Hoff plot for bombesin derived from $\log \bar{k}$ values at $\bar{\psi} = 0.3$ on the C_{18} phase demonstrates a biphasic relationship, with two distinct regions within the curve. These regions correspond to segments at 5–55 and 65–85°C, with an apparent transition around 60°C. The ΔS^0_{assoc} values derived from these two regions were 6.2 and -47.5 J mol^{-1} K^{-1} respectively. At the higher temperatures a more negative change in entropy is observed. These results also indicate that the molecule is more flexible in solution at temperatures above 60°C than when bound to the stationary phase ligands. However, below this temperature the molecule is not significantly more flexible in solution than when bound, suggesting that bombesin may possess a more rigid solution structure at the lower temperatures. These data also suggest that the increased organic modifier concentration may be stabilizing the bombesin secondary structure, as is evident from the increased transition temperature observed at the higher organic modifier concentration. This finding correlates well with other physico-chemical studies on bombesin using NMR and circular dichroism spectroscopy [8,9,23], where it has been found that bombesin exists in a helical conformation in the presence of organic solvents such as trifluoroethanol or lipid micelles.

Bombesin on C_4 phase. The Van 't Hoff plot for bombesin at extrapolated values of $\log \bar{k}$ at $\bar{\psi} = 0.1$ on the C_4 phase was essentially linear, whereas on the C_{18} phase a transition at *ca.* 25°C was observed. The ΔS^0_{assoc} value derived for these conditions was -4.5 J mol^{-1} K^{-1}, indicating little change in the flexibility between the bound and unbound bombesin molecule. For $\log \bar{k}$ ($\bar{\psi} = 0.3$) a biphasic relationship exists for the Van 't Hoff plot, with two distinct regions corresponding to segments at 5–45°C and 45–85°C. The ΔS^0_{assoc} values derived from these two regions were -5.2 and -94.4 J mol^{-1} K^{-1}, respectively. These values reflect a transition from a rigid or conformationally constrained solute molecule to a more flexible structure. The transition

at *ca.* 45°C is lower than the observed transition at 60°C for the C_{18} stationary phase.

These results indicate that the C_{18} ligands are more capable of stabilizing a "low-temperature" peptide conformational structure, which is either not present with the C_4 ligands or the transition for the same structure on the C_4 phase is shifted towards lower temperatures. If the experimental results with the C_4 and C_{18} ligands are compared, the latter possibility appears to be more likely, that is, the conformational transition is moved to a lower temperature on the C_4 phase, as is observed for the transition temperatures at log $\bar{k}$ ($\bar{\psi} = 0.3$). This finding suggests that the C_{18} phase can enhance the stability of the solute secondary structure. Similar results have been observed with the binding of enzymes to agarose gels substituted with *n*-alkyl chains and weakly hydrophobic silica sorbents [24, 25].

β-Endorphin on C_{18} phase. Fig. 7 represents Van 't Hoff plots for β-endorphin chromatographed on both C_{18} and C_4 phases. The Van 't Hoff relationship for log $\bar{k}$ values determined at $\bar{\psi} = 0.1$ for the solute chromatographed on the C_{18} stationary phase is non-linear with a transition occurring around 50°C. Two distinct regions of the curve, namely 5–45 and 45–85°C, are apparent. The lower temperature region yields a positive ΔS^0_{assoc} value of 30.7 J mol^{-1} K^{-1}. This result indicates that the molecules become more disordered on binding to the stationary phase, reflecting a static solution structure at these temperatures, which becomes less ordered on adsorption. At the elevated temperatures, a negative ΔS^0_{assoc} value of -16.0 J mol^{-1} K^{-1} was derived for β-endorphin from the Van 't Hoff plot. This result reflects a more flexible high-temperature solution structure for β-endorphin compared with the lower temperature structure, and this high-temperature structure becomes more constrained on binding to the stationary phase, resulting in an overall increase in the order of the system. Perturbation of the solution structure of the molecule would thus occur at the higher temperatures, a conclusion consistent with the gradual increase in S values observed with increasing temperature for this solute when chromatographed on the C_{18} phase.

The Van 't Hoff plots for β-endorphin chromatographed on the C_{18} stationary phase at log $\bar{k}$ values determined at $\bar{\psi} = 0.3$ gave a non-linear relationship with an apparent transition at 50°C, similar to that observed for the low organic mole fraction condition on this phase. In this instance two distinct regions of the Van 't Hoff curve were again apparent, at 5–45 and 45–85°C. The ΔS^0_{assoc} value derived from the lower temperature region was small and negative (-7.3 J mol^{-1} K^{-1}), which is in contrast to the positive ΔS^0_{assoc} value observed at the lower organic modifier concentration for this temperature range. Hence the increased concentration of organic modifier seems to make the molecule more flexible in solution at these temperatures. At higher temperatures, the derived ΔS^0_{assoc} value was large and negative (-36.6 J mol^{-1} K^{-1}), and is also more negative than the observed value at the lower organic modifier concentration. Thus, again it would appear that solute is more flexible in the higher organic modifier concentration. It would also appear that the system is more disordered at higher organic mole fractions, indicating that the secondary structure of the β-endorphin solute is less well defined at the higher organic solvent concentration. This behaviour is in contrast to that observed for bombesin, which appeared to be stabilized by the increased organic modifier concentration, and may represent solvation of residues normally involved in intramolecular interactions. This possible structural change could then explain the observed increase in disorder illustrated by the ΔS^0_{assoc} values obtained for β-endorphin.

β-Endorphin on C_4 phase. Van 't Hoff plots for β-endorphin chromatographed on the C_4 phase are also displayed in Fig. 7. For the Van 't Hoff plot of log $\bar{k}$ values determined at $\bar{\psi} = 0.1$, the curve has two distinct regions, at 5–55 and 55–85°C, with an apparent transition around 55°C, a value similar to the observed transition at $\bar{\psi} = 0.1$ on the C_{18} phase. ΔS^0_{assoc} values were derived from both regions of the Van 't Hoff plot and at the lower temperature range a ΔS^0_{assoc} value of -18.3 J mol^{-1} K^{-1} was obtained. This behaviour reflects a total increase in ordering of the system on adsorption to the stationary phase, and thus indicates a flexible solution structure. Log $\bar{k}$ at the higher temperature range yielded a ΔS^0_{assoc} value of -180.0 J mol^{-1} K^{-1}, which indicates a large increase in ordering of the system on adsorption and reflects a very flexible solution structure at these elevated temperatures. A much larger relative increase in ordering occurs under these C_4 condi-

tions compared with the C_{18} phase, and can be attributed to the more rigid structure of the C_4 ligand [17–19].

The Van 't Hoff plots for β-endorphin chromatographed on the C_4 phase, with log $\bar{k}$ values at $\bar{\psi}$ = 0.3, showed three distinct regions, 5–45, 45–55 and 55–85°C, with an apparent transition at 50°C. This behaviour is similar to the lower organic mole fraction transition temperature on this phase, and also similar to both transition temperatures observed on the C_{18} phase. Hence it would appear that the ligand hydrophobicity does not affect the observed transitions for β-endorphin, unlike the observed stabilization of bombesin on the C_{18} phase noted earlier. The ΔS^0_{assoc} value derived from the lowest temperature region (5–45°C) was −15.2 J mol^{-1} K^{-1}. This value reflects an overall increase in order of the system on adsorption, and thus reflects a more flexible molecule in solution than when associated with the stationary phase. At the transition region (45–55°C) a ΔS^0_{assoc} value of −82.4 J mol^{-1} K^{-1} was obtained, indicating an increased flexibility in solution at these temperatures relative to the lower temperatures. In contrast, at the higher temperatures a ΔS^0_{assoc} value of −4.8 J mol^{-1} K^{-1} was derived. This value suggests that the molecule is least flexible in solution at these high temperatures or, more likely, that a rapidly interconverting time-averaged structure is being observed. Such a structure would undergo rapid conformational interconversions both in solution and when bound, hence the relative change in the ordering of the system would be relatively small for the association process.

Unlike the results on the C_{18} phase for this solute, the magnitude of ΔS^0_{assoc} on the C_4 phase at $\bar{\psi}$ = 0.1 is larger and more negative than the corresponding value at $\bar{\psi}$ = 0.3. This behaviour indicates that there is a larger increase in ordering on adsorption in the presence of a lower organic mole fraction for this solute.

Glucagon on C_{18} phase. Fig. 8 displays Van 't Hoff plots for glucagon chromatographed on both C_{18} and C_4 phases at the two extrapolated values of $\bar{\psi}$. The Van 't Hoff relationship for log $\bar{k}$ values at $\bar{\psi}$ = 0.1 is non-linear for glucagon chromatographed on the C_{18} phase. The Van 't Hoff curve is biphasic, possessing two distinct regions, at 5–45 and 45–85°C, with an apparent transition around 50°C. The ΔS^0_{assoc} values derived from these two regions were 26.1 and −23.7 J mol^{-1} K^{-1}, respectively. Again, the decrease in ordering of the system on adsorption at the lower temperatures can be noted, indicating a stable solution structure which becomes more flexible on interaction with the stationary phase. At the elevated temperatures an overall increase in ordering of the system is apparent, reflecting a more flexible solution structure relative to the low-temperature structure. Thermal disruption of solution structure and hence a gradual increase in the S value and hence chromatographic contact area observed on this phase would account for this behaviour.

The Van 't Hoff relationship for log $\bar{k}$ values determined at $\bar{\psi}$ = 0.3 was also biphasic, with two distinct regions spanning 5–45 and 45–85°C, and an apparent transition at around 50°C. This value is similar to the transition temperature observed for the lower organic modifier concentration. The ΔS^0_{assoc} values derived for the lower and higher temperature regions were −13.0 and −47.4 J mol^{-1} K^{-1}, respectively. Again, this result indicates a flexible molecule in solution at both temperature ranges, with an overall increase in ordering on association with the stationary phase in both instances. The relatively larger increase in ordering at the higher temperatures suggests a more flexible molecule than at the lower temperatures, again indicating possible disruption of the solution structure at the higher temperatures. Further, the influence of increased organic mole fraction appears to be the increased flexibility in solution of the solute molecule, as was observed for β-endorphin on the C_{18} phase.

Glucagon on C_4 phase. The Van 't Hoff plot for glucagon chromatographed on the C_4 phase at log $\bar{k}$ values determined at $\bar{\psi}$ = 0.1 displays a biphasic relationship. The curve consists of two regions spanning 5–45 and 45–85°C, with a transition around 50°C, which is similar to the transitions observed for glucagon on the C_{18} phase. Ligand hydrophobicity therefore does not seem to affect the observed transition temperature, as was also observed for β-endorphin. The ΔS^0_{assoc} values derived from these two regions were −97.6 and −169.0 J mol^{-1} K^{-1}, respectively. These results indicate a considerably flexible structure at low temperatures that increases in flexibility with increasing temperature. In both instances the association process involves significantly large increases in the ordering

of the system, with a larger degree of ordering at the higher temperatures, which reflects changes in the solution structure of glucagon. As was evident with the other peptidic solutes, the magnitude of the increase in ordering of the system on adsorption was much larger on the C_4 phase than the C_{18} phase.

The Van 't Hoff plot for glucagon determined for log $\bar{k}$ values at $\bar{\psi} = 0.3$ gives a curve that can be divided into three regions, 5–25, 25–75 and 75–85°C. The ΔS^0_{assoc} values derived from these temperature regions were 2.1, −45.4 and 12.6 J mol^{-1} K^{-1}, respectively. These values suggest an initially rigid molecule undergoing a transition around 50°C which corresponds to an increase in ordering on association with the stationary phase and indicates that at these temperatures the molecule is flexible in solution. At the higher temperatures a time-averaged structure is observed with rapid conformational interconversion both in solution and when bound to the stationary phase explaining the positive ΔS^0_{assoc} value obtained.

The difference in magnitude of the ΔS^0_{assoc} for the two organic modifier concentrations on the butyl stationary phase demonstrate a general trend of the ΔS^0_{assoc} values being more negative at $\bar{\psi} = 0.1$. The behaviour of glucagon is thus similar to the corresponding case with β-endorphin. It can be concluded from these observations that glucagon is characterized by a higher degree of flexibility in solution at the lower organic modifier concentration on the C_4 phase.

CONCLUSIONS

Overall, the thermodynamic data for bombesin, β-endorphin and glucagon illustrate transitions in the 50–60°C region, suggesting that the interactive structure is dramatically disrupted over this temperature range. This value is also the temperature range over which helices tend to be disrupted, reiterating the hypothesis that the amphipathic helical portions of these molecules are either directly involved in the interaction between the stationary phase ligands or are able to stabilize the peptide surface exposed to the stationary phase ligands. When these helical conformations are perturbed, the corresponding change in the hierarchical structure and the interactive surface presented to the stationary phase will result in the observed changes in retention behaviour.

The differences in retention and thermodynamic parameters derived for each solute and the differing temperature dependencies of these parameters clearly demonstrate that the peptide sequence plays a crucial role in the interactive properties of peptide solutes. In this study, bombesin exhibited retention behaviour which was generally distinct to that observed with β-endorphin and glucagon. Although β-endorphin and glucagon are larger molecules than bombesin, it is not the molecular size *per se* that controls the chromatographic behaviour, but rather the ability of larger molecules to stabilize their secondary structure without external influences.

For both β-endorphin and glucagon the ΔS^0_{assoc} values are all more negative at the higher temperatures, that is, the peptides are more flexible in solution at higher than at lower temperatures. The large negative value of ΔS^0_{assoc} would suggest that on binding to the hydrocarbonaceous ligands the number of available conformations of the polypeptide dramatically decreases. The ΔS^0_{assoc} values for these peptides for the postulated melted structure (*i.e.*, the high temperature structures) were generally more negative for the C_4 phase than the corresponding values derived from the C_{18} stationary phase. This observation means there is a larger relative increase in the structural ordering of the solutes on their adsorption to the C_4 phase than to the C_{18} phase, that is, the peptide is restricted to fewer conformations when bound to the ligand than for the corresponding interaction with the C_{18} ligand. This is consistent with the proposal that the C_4 ligand is rigid as opposed to the more flexible C_{18} ligands.

The change in enthalpy follows the same trends as seen for the changes in entropy. These changes are presumably a consequence of related changes in solvation energies as the entropically driven changes in the solute structure occur.

The factors which stabilize α-helical or any other secondary structure of a polypeptide include sequence-specific intramolecular charged interactions, hydrogen bonding and also the amphipathic arrangement of polar and non-polar residues along the peptide sequence. The ability to monitor changes in these stabilizing influences by employing hydrophobic stationary phases and varied temperature gives an insight into the underlying principles of peptide folding and peptide–surface interactions. In this investigation the influence of temperature on the

chromatographic behaviour of several peptide solutes was examined. The temperature range covered the region where secondary structure is normally perturbed. The three peptidic hormone solutes studied have a major portion of their structure stabilized by α-helices at ambient temperature [8–13]. This study has shown that conformational transitions, such as the melting of the solution structure of the peptide, may be observed by utilizing chromatographic techniques. It is clear that the two different ligands studied have different abilities to monitor conformational changes of the solute. This discriminatory ability may be due either to differences in stabilizing forces associated with the ligand–solute interaction or to the solvated structure of the ligand themselves. Structurally disparate ligand surfaces would be expected to interact with different mechanisms [17], which would then be manifested as different dependencies of S and $\log k_0$ on temperature. Similar conclusions have been reached from evaluating peptide–ligand interactions by principle component analysis [18]. Generally, it appears that the C_{18} stationary phase tended to stabilize interactive structures relative to the C_4 stationary phase. In addition, increasing the organic modifier concentration resulted in destabilization of the interactive secondary structures of β-endorphin and glucagon, but stabilization of the bombesin secondary structure. The molecular basis of this interaction requires further analysis of the solution conformation and dynamics of these peptides, and is the subject of ongoing investigations.

ACKNOWLEDGEMENTS

These investigations were supported by grants from the Australian Research Council, the National Health and Medical Research Council of Australia and the Monash University Research Grants Committee.

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