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Temperature effects in hydrophobic interaction chromatography

(thermodynamics/hydrophobic effect/molecular chromatography)

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ABSTRACT **The effect of temperature from 5°C to 50°C on the retention of dansyl derivatives of amino acids in hydrophobic interaction chromatography (HIC) was investigated by HPLC on three stationary phases. Plots of the logarithmic retention factor against the reciprocal temperature in a wide range were nonlinear, indicative of a large negative heat capacity change associated with retention. By using Kirchoff's relations, the enthalpy, entropy and heat capacity changes were evaluated from the logarithmic retention factor at various temperatures by fitting the data to a logarithmic equation and a quadratic equation that are based on the invariance and on an inverse square dependence of the heat capacity on temperature, respectively. In the experimental temperature interval, the heat capacity change was found to increase with temperature and could be approximated by the arithmetic average. For HIC retention of a set of dansylamino acids, both enthalpy and entropy changes were positive at low temperatures but negative at high temperatures as described in the literature for other processes based on the hydrophobic effect. The approach presented here shows that chromatographic measurements can be not only a useful adjunct to calorimetry but also an alternative means for the evaluation of thermodynamic parameters.**

Hydrophobic interaction chromatography (HIC) is widely used for the separation and purification of proteins in their native state (1, 2). The technique employs weakly hydrophobic stationary phases and the retention is modulated by varying the salt concentration in the aqueous mobile phase (3, 4). The effect of salt on protein interactions in aqueous solutions (5–9) as well as on protein adsorption in HIC (5, 10, 11) has been extensively investigated, and the salt effect on HIC retention has been treated within the hermeneutics of the solvophobic theory (12–14) and Wyman's linked functions (15–18).

Less progress has been made in elucidating the effect of temperature on retention in HIC. The observed increase in protein retention with temperature, for instance, has been ascribed to enhancement of hydrophobic interactions with increasing temperature due to temperature-induced conformational changes of proteins and concomitant increase in hydrophobic contact area upon binding to the chromatographic surface (16). So far the only thermodynamic study on the effect of temperature in HIC was carried out with aliphatic alcohols and carboxylic acids on octyl-agarose stationary phase with neat aqueous phosphate buffers (19) and hydro-organic mobile phases containing methanol or ethylene glycol (20). Since the salt concentration was very low in these studies, the conditions differed from those employed for protein separation in HIC. In order to shed light on the HIC retention behavior of proteins, it is necessary to understand first the physicochemical basis of HIC with simple molecules that undergo no significant conformational changes and to use conditions that simulate those employed for protein separation.

Retention and selectivity in HIC are likely to be governed by the hydrophobic effect (21). The study of the thermodynamic aspects of hydrophobic interactions, which are believed to determine also the three-dimensional architecture, stability, and dynamics of protein molecules (22), has been a long-standing goal of calorimetric studies on protein folding (23–30). More often than not, relatively simple model systems, such as dissolution of gaseous, liquid, and solid compounds in water, have been employed to evaluate the thermodynamic quantities associated with hydrophobic interactions. Calorimetric studies on the transfer of liquid hydrocarbons (31), nonpolar gaseous substances (32, 33), and solid cyclic dipeptides (34) to water has revealed significant heat capacity effects. This behavior is attributed primarily to removal of nonpolar surface area of the molecule from water upon transfer (35, 36). Data obtained in such model experiments has therefore provided the basis of a more detailed understanding of the influence of temperature on hydrophobic interactions and of the hydrophobic effect at large. Since retention in HIC entails a similar transfer process, the above model studies are expected to facilitate the analysis of the temperature effect in this technique.

In the present work the retention of dansylamino acids is investigated under conditions of isocratic salt elution on three different HIC columns. Unlike in preparative or analytical applications, chromatography is used here to extract physicochemical and molecular information from retention data. The thermodynamic approaches, which have been employed in the analysis of the processes mentioned above, serve as a framework for the interpretation of HIC retention data.

THEORY

The magnitude of retention of a substance, conveniently called the eluite in linear elution chromatography, is measured under isocratic condition by the retention factor, k' (37):

$$k' = \frac{V_r - V_o}{V_o}, \qquad [1]$$

where V_r and V_o are the retention volumes of the eluite and of an "inert" tracer. Under isorheic conditions k' is evaluated from the volumetric flow rate and the retention times on the chromatogram.

The distribution of the eluite between the bulk mobile phase and the stationary phase is determined by the standard free energy change, $\Delta G°$, associated with eluite transfer from the mobile to the stationary phase. The corresponding equilibrium constant, K, is given by

$$K = e^{-\Delta G°/RT} \qquad [2]$$

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Abbreviation: HIC, hydrophobic interaction chromatography.

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and related to k' (37):

$$k' = K\phi, \quad [3]$$

where ϕ is the phase ratio—i.e., the volume ratio of the stationary phase and the mobile phase in the column, which is believed to be independent of temperature. Combination of Eqs. **2** and **3** yields

$$\ln k' = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi, \quad [4]$$

where ΔH° and ΔS° are the standard enthalpy and entropy changes for eluite transfer from the mobile phase to the stationary phase. When enthalpy and entropy changes are temperature invariant, plots of $\ln k'$ versus $1/T$ (van't Hoff plots) are, according to Eq. **4**, linear and allow their direct evaluation, provided that the phase ratio is known.

The measured thermodynamic quantities associated with the retention are believed to represent average values due to the heterogeneity of the chromatographic surface. Yet retention data may yield linear van't Hoff plots, provided that the pertinent surface, eluite, and solvent properties are temperature invariant. Eluite molecules in this investigation are not expected to change with temperature. Similarly, the density of nonpolar bonded chains of the stationary phase and their accessibility to eluites, and thus the contact surface area upon binding, remain temperature invariant.

When ΔH° and ΔS° are temperature dependent, the integrated form of Kirchoff's law is used to evaluate the thermodynamic quantities (23–26). If the heat capacity change, ΔC_p°, is invariant with temperature, the dependence of ΔH° and ΔS° on the experimental temperature is given by

$$\Delta H^\circ = \Delta C_p^\circ (T - T_H) \quad [5]$$

and

$$\Delta S^\circ = \Delta C_p^\circ \ln(T/T_S), \quad [6]$$

where T_H and T_S are reference temperatures at which ΔH° and ΔS° are zero. Combining Eqs. **4, 5** and **6** we obtain for the temperature dependence of the logarithmic retention factor the expression

$$\ln k' = \frac{\Delta C_p^\circ}{R}\left(\frac{T_H}{T} - \ln\frac{T_S}{T} - 1\right) + \ln \phi, \quad [7]$$

which is termed the "logarithmic equation." Eq. **7** allows the evaluation of the three parameters from nonlinear van't Hoff plots by a least-squares fitting procedure if the phase ratio of the column is known or can be estimated. With the parameters from Eq. **7** the enthalpy and entropy changes are readily evaluated by Eqs. **5** and **6** for any temperature within the experimental range. Temperature-invariant heat capacity change is a crude assumption. Very recently calorimetric data on protein folding in a wide enough temperature range has shown that the heat capacity change is dependent on the temperature and converges to zero at a sufficiently high temperature (29, 30).

For this reason, we also evaluated the thermodynamic quantities associated with the chromatographic retention process without assuming constant ΔC_p° by using the three-parameter expression

$$\ln k' = a + \frac{b}{T} + \frac{c}{T^2} + \ln \phi, \quad [8]$$

which is termed the "quadratic equation." Eq. **8** was found applicable with retention data in reversed-phase chromatography at temperatures from −5°C to 80°C and within the accuracy of the measurements (38). By fitting Eq. **8** to the experimental data its parameters are evaluated and used to calculate the enthalpy change as

$$\Delta H^\circ = -R\frac{d\ln k'}{d(1/T)} = -R\left(b + \frac{2c}{T}\right). \quad [9]$$

The corresponding entropy change is

$$\Delta S^\circ = R\left(a - \frac{c}{T^2}\right), \quad [10]$$

and the temperature dependence of the heat capacity is given by

$$\Delta C_p^\circ = \frac{2Rc}{T^2}. \quad [11]$$

Whereas the logarithmic equation is based on constant ΔC_p°, the quadratic equation allows for its temperature dependence and may gain greater significance because recent calorimetric studies on protein folding have shown quite appreciable changes in heat capacity with temperature (29, 30, 39). The logarithmic equation is expected to yield an average value for ΔC_p° in the experimental temperature range. This will be compared with arithmetic mean value of heat capacity changes obtained by the quadratic equation.

METHODS

Instrumentation. A series 400 solvent delivery system and a model LC-95 UV/VIS detector (Perkin Elmer, Norwalk, CT) with a model C-R3A Chromatopac integrator (Shimadzu) were used in chromatographic experiments. The column, a heat exchanger coil made of a 1-m-long capillary tube (1/16th-inch o.d., 0.5-mm i.d.), and a model 7125 Rheodyne injection valve (Cotati, CA) were immersed in a model D8L circulating water bath (Haake, Berlin) of 3-liter capacity with the inlet and outlet of the circulating system shunted. The accuracy of the Wattomatic temperature control was ±0.02°C. For experiments at subambient temperatures, coolant from a model RTE-4DD refrigerated circulating bath (Neslab Instruments, Porthmouth, NH) was circulated through the built-in cooling coil of the Haake bath. Chromatographic experiments were carried out after thermal equilibrium was reached. The temperature of the column and the water in the thermostatted bath were taken to be equal.

HIC Columns. Spherogel HIC, 100 mm × 4.6 mm (A), SynChropak propyl, 100 mm × 4.6 mm (B), and TSK-GEL butyl-NPR, 35 mm × 4.6 mm (C), columns were used until their retentive properties remained invariant. Column A was unstable above 45°C whereas columns B and C were stable at temperatures up to 50°C. As seen in Table 1, columns A and B were packed with porous bonded siliceous stationary phases

Table 1. Properties of stationary phases employed in the study of the effect of temperature on the retention in HIC

Column	Functional groups	Support
(A) Spherogel HIC	Polyethylene glycol with methoxy end groups	300-Å porous spherical silica, (d_p = 5 μm)
(B) SynChropak propyl	Propyl ligates bound to crosslinked polyethyleneimine	300-Å porous spherical silica (d_p = 6.5 μm)
(C) TSK-GEL butyl-NPR	Butyl ligates attached to bonded hydrophilic layer	Nonporous crosslinked acrylic microbeads (d_p = 2.5 μm)

whereas column C was packed with a micropellicular-type stationary phase consisting of fluid impervious acrylic microspheres with a retentive layer.

Chemicals. Reagent-grade ammonium sulfate, sodium sulfate, dibasic sodium phosphate, and 85% phosphoric acid were obtained from J. T. Baker (Phillipsburg, NJ). Glycylglycine and dansyl derivatives of amino acids were purchased from Sigma. The codes for the amino acid moieties are as follows: Gly (glycine), Ala (alanine), Abu (α-amino n-butyric acid), γAbu (γ-aminobutyric acid), Nva (norvaline), Val (valine), Pro (proline), Leu (leucine), Nle (norleucine), Phe (phenylalanine), Met (methionine), and Tyr (tyrosine).

Mobile Phases. Deionized HPLC-grade water obtained with a NanoPure water purification system (Barnstead, Boston) was used to prepare eluents having the desired molar concentrations of ammonium or sodium sulfate as well as dibasic sodium phosphate. The pH of the solution was adjusted to 7.0 with 85% phosphoric acid. The solution was filtered through a 0.45-μm Nalgene filter (Nalge) and purged with helium prior to use.

Procedures. Prior to injection the column was brought to the operating temperature and equilibrated with the mobile phase preheated to column temperature. Dansylamino acids were dissolved in the mobile phase at concentrations of about 0.01 mg/ml. The injection volume varied between 5 and 10 μl. Dansylamino acids were eluted isocratically at temperatures varying from 5°C to 50°C and detected at 215 nm. Gradient elution was used only for the separation depicted in Fig. 1. Glycylglycine was used as the inert tracer, and V_o and the retention volumes (V_r) of the dansylamino acids were measured at each mobile phase composition and temperature in duplicate. The V_o of the column did not depend on the salt concentration in the mobile phase.

RESULTS AND DISCUSSION

HIC is used mainly for the separation of biological macromolecules—above all, proteins. The size, complex structure, and tendency to denaturation of proteins make it rather difficult to explicate the effect of temperature on the energetics of the retention process in HIC with proteinaceous eluites. For this reason we used as model compounds dansylamino acids, which are relatively simple molecules with sizeable hydrophobic moieties, not expected to undergo on-column conformational changes under conditions used in HIC, retained sufficiently, and detected at low concentrations due to their strong UV absorption. Fig. 1 shows a typical chromatogram of six dansylamino acids that was obtained by HIC with gradient elution under conditions similar to those in protein separations.

The retention factors of selected dansylamino acids were measured in the temperature range from 5°C to 45°C on column A and from 5°C to 50°C on columns B and C. By setting arbitrarily the value of the phase ratio to unity, the data were fitted according to the logarithmic and quadratic equations—i.e., Eqs. **7** and **8**—with $r^2 \geq 0.995$. Fig. 2 illustrates the best fits of the logarithmic equation to the data obtained with the three columns. The van't Hoff plots are highly nonlinear and, with a few exceptions, go through a maximum in the experimental temperature interval. This behavior is indicative of a large heat capacity change accompanying the retention process in HIC (21).

The thermodynamic quantities associated with the chromatographic process were extracted from the temperature dependence of the retention on the three columns by using the logarithmic and quadratic equations, and the results are illustrated in Table 2 for the retention of dansylamino acids on column A at various temperatures. The assumption of $\phi = 1$ affects only the value of entropy among the above thermodynamic quantities according to Eqs. **7** and **8**. The phase ratio is believed to decrease with increasing column temperature at a rate of 0.1% per 10°C. Even in the worst case, for a 10% change in ϕ over the temperature range 5–50°C the respective errors in ΔC_p° and ΔG° are estimated to be no higher than 3% and 5%. The errors in ΔH° can be significantly higher but less than 25% except for ΔH° values close to zero.

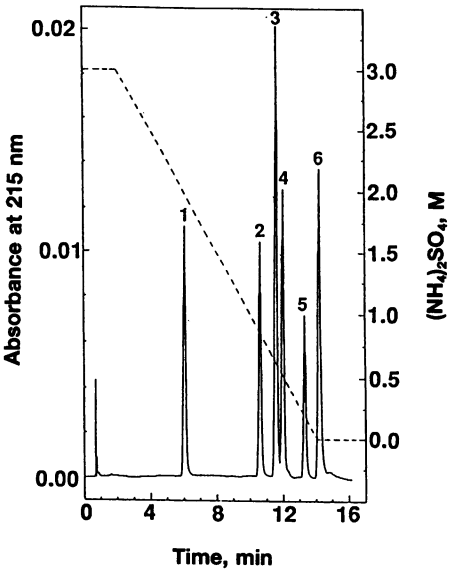

FIG. 1. Typical chromatogram of dansylamino acids. Column A, 100 mm × 4.6 mm, packed with 5-μm Spherogel HIC; 12-min linear gradient from 3 M ammonium sulfate in 0.1 M sodium phosphate buffer, pH 7.0, to the neat buffer; flow rate, 1.5 ml/min; temp., 20°C; UV detection at 215 nm. Sample components: dansyl derivatives of His, (peak 1), Ala (peak 2), Val (peak 3), Leu (peak 4), Phe (peak 5), and Tyr (peak 6).

The ΔH°, ΔS°, and ΔG° values obtained by the two approaches at any given temperature are within 10% of each

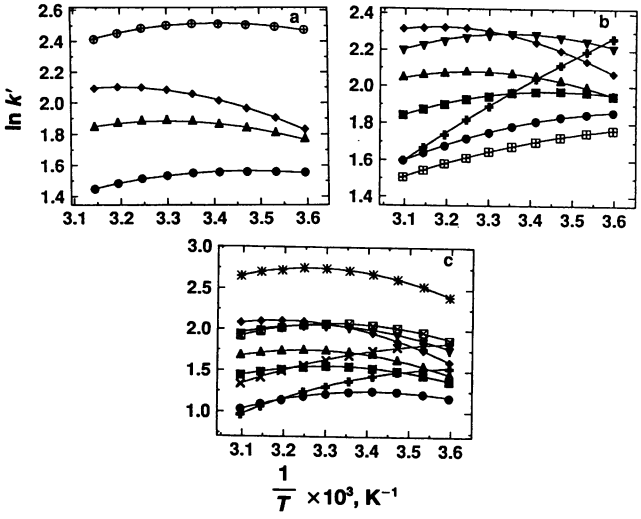

FIG. 2. van't Hoff plots of retention data obtained with dansylamino acids on three HIC columns. (*a*) Column A, 100 mm × 4.6 mm, packed with 5-μm Spherogel HIC; mobile phase, 1.25 M ammonium sulfate in 0.05 M sodium phosphate, pH 7.0; flow rate, 1.5 ml/min. (*b*) Column B, 100 mm × 4.6 mm, packed with 6.5-μm SynChropak propyl; mobile phase, 0.7 M ammonium sulfate in 0.05 M sodium phosphate, pH 7.0; flow rate, 2.0 ml/min. (*c*) Column C, 35 mm × 4.6 mm, packed with 2.5-μm TSK-GEL butyl-NPR; mobile phase, 1.25 M ammonium sulfate in 0.05 M sodium phosphate, pH 7.0; flow rate, 1.0 ml/min. Sample components: dansyl derivatives of Gly (✚), Ala (●), Abu (■), Nva (▼), Val (▲), Leu (◆), γAbu (✖), Phe (⊕), Pro (⊞), Nle (✳), and Met (⊠). Solid lines represent the best fits of Eq. **7** to the data.

Table 2. Temperature dependence of thermodynamic quantities associated with the retention of dansylamino acids on Spherogel column as evaluated by fitting the logarithmic equation, Eq. **7** (L), and the quadratic equation, Eq. **8** (Q), to experimental data

Amino acid moiety	Temp., °C	ΔC_p°, cal·mol^{-1}·K^{-1}		ΔH°, cal·mol^{-1}		ΔS°, cal·mol^{-1}·K^{-1}		ΔG°, cal·mol^{-1}	
		L	Q	L	Q	L	Q	L	Q
Ala	5		−52.8	438	481	4.7	4.8	−860	−857
	15		−49.2	−26	−26	3.0	3.0	−899	−894
	25		−45.9	−490	−503	1.4	1.4	−921	−918
	35		−43.0	−954	−948	−0.1	−0.1	−928	−924
	45		−40.3	−1417	−1364	−1.6	−1.4	−920	−917
	5–45	−46.4	−46.1*						
Val	5		−69.8	1538	1592	9	9.2	−975	−976
	15		−65.1	924	918	6.9	6.8	−1055	−1056
	25		−60.8	310	289	4.8	4.7	−1113	−1113
	35		−56.9	−304	−299	2.7	2.8	−1150	−1150
	45		−53.4	−918	−851	0.8	1.0	−1168	−1169
	5–45	−61.4	−61.1*						
Leu	5		−83.9	2591	2656	12.9	13.2	−1006	−1010
	15		−78.2	1854	1846	10.3	10.3	−1123	−1127
	25		−73.0	1117	1091	7.8	7.7	−1213	−1218
	35		−68.4	380	384	5.4	5.4	−1279	−1283
	45		−64.1	−357	−278	3.0	3.3	−1321	−1327
	5–45	−73.7	−73.3*						
Phe	5		−71.4	962	1021	8.4	8.6	−1367	−1366
	15		−66.5	335	332	6.2	6.1	−1440	−1439
	25		−62.1	−292	−310	4.0	4.0	−1491	−1489
	35		−58.1	−919	−911	2.0	2.0	−1520	−1519
	45		−54.6	−1546	−1474	0.0	0.2	−1530	−1529
	5–45	−62.7	−62.4*						

Experimental conditions were as in Fig. 2*a*.
*Mean value of heat capacity, $\overline{\Delta C_p^\circ}$.

other. The heat capacity changes in the temperature range 5–45°C were evaluated by using the quadratic equation and for each eluite the values of the arithmetic mean heat capacity, $\overline{\Delta C_p^\circ}$, were calculated. Table 2 shows excellent agreement between $\overline{\Delta C_p^\circ}$ and the corresponding ΔC_p° obtained by using the logarithmic equation. The results of the two fitting procedures show that even if the heat capacity change is dependent on the temperature in HIC, it may be assumed constant and represented by the arithmetic mean heat capacity change in a sufficiently narrow temperature interval.

Fig. 3 illustrates the temperature dependence of ΔC_p° according to Eq. **10**. The ΔC_p° values are negative and decrease in magnitude with increasing temperature for all dansylamino acids in a linear fashion. The more hydrophobic a dansylamino acid is, the steeper is the rise of ΔC_p° with the temperature. The linearity is expected from the data in Table 2 that show the

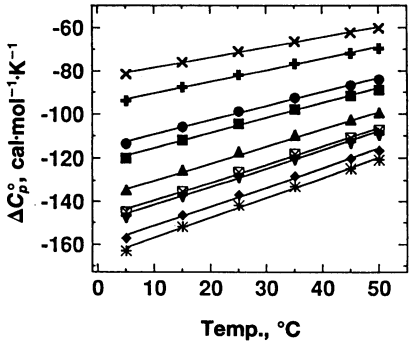

FIG. 3. Temperature dependence of the heat capacity change associated with the retention of dansyl amino acids on TSK-GEL butyl-NPR HIC column as obtained from the fit of the quadratic equation to the experimental retention data. Solid lines represent linear least-squares fits to the data. Symbols are as in Fig. 2.

arithmetic mean heat capacity changes to be almost the same as the heat capacity changes obtained by Eq. **8** at 25°C, which is the corresponding arithmetic mean temperature. By extrapolation of the data obtained on all three columns, the lines converge to zero at about 175°C. By and large, ΔC_p° values for the dansylamino acids investigated in this study vary not more than ±15% of the arithmetic mean values in the experimental temperature range, in agreement with calorimetric studies on protein folding (30). Comparison of chromatographic data to precision calorimetric measurements with appropriate chromatographic sorbents will be required, however, to confirm the accuracy and usefulness of this approach.

As seen from Table 2 both ΔH° and ΔS° strongly depend on the temperature. They are positive at low temperatures, decrease with increasing temperature, and become negative at high temperatures. In contradistinction, ΔG° and ΔC_p° are negative and exhibit a much weaker dependence on the temperature. Data measured with all three HIC columns showed similar behavior. This demonstrates that the retention in HIC is entropy-driven at low temperatures and enthalpy-driven at high temperatures, as in other processes governed by the hydrophobic effect (24).

In Fig. 4 the thermodynamic quantities associated with the retention on the three columns at 25°C of dansylamino acids which vary only in the length of the aliphatic side chain are plotted against the carbon number of the side chain. The plots in Fig. 4 show that ΔG° and ΔC_p° values become more negative with increasing hydrophobic properties of the dansylamino acids. On the other hand, ΔH° and ΔS° exhibit the opposite trend, becoming more positive with the size of the eluite. The plots are reasonably linear, suggesting that the thermodynamic quantities can be expressed in terms of the nonpolar surface area in these molecules, which is proportional to the carbon number of the side chain.

The effect of the stationary phase on the thermodynamic quantities can also be gleaned from Fig. 4. The heat capacity

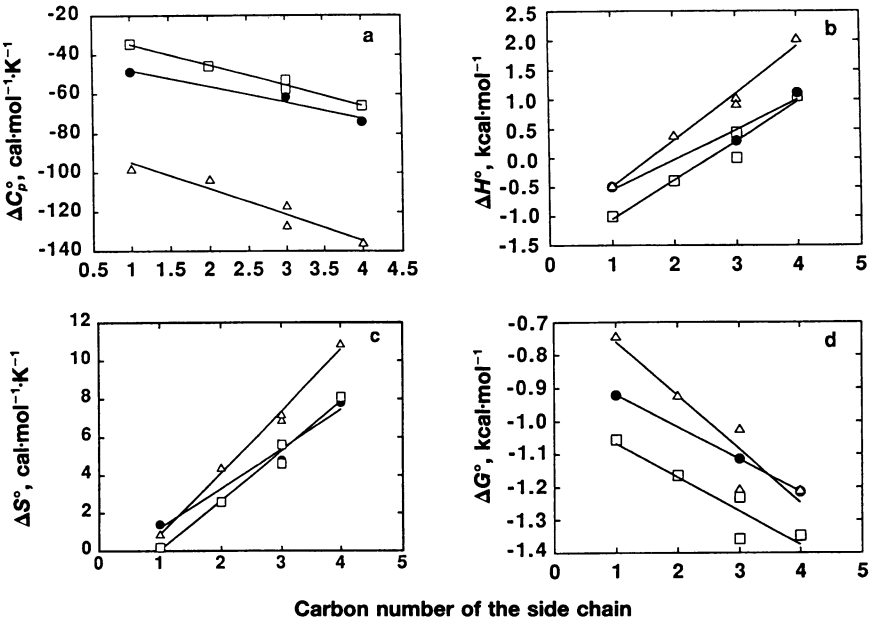

FIG. 4. Plots of the heat capacity (*a*), enthalpy (*b*), entropy (*c*), and free energy (*d*) against the carbon number of the side chain of the amino acid moiety for the retention of dansylamino acids on Spherogel (●), SynChropak propyl (□) and TSK-GEL butyl-NPR (△) HIC columns at 25°C.

change is significantly smaller for the process on columns A and B than for the process on column C. On column B this is probably the result of electrostatic interactions between positively charged amino groups, known to exist on the surface of this stationary phase, and the anionic moieties of the dansylamino acids (14). Polar interactions tend to reduce the magnitude of the heat capacity change—i.e., make it less negative (34, 40). This could also explain the effect observed on column A, which had fixed polyethylene glycol functions at the chromatographic surface that may also partake in polar interactions. On the other hand, column C had highly nonpolar *n*-butyl chains attached to the hydrophilized surface of the stationary phase so that polar interactions most likely play a lesser role in the retention process.

CONCLUSIONS

The scope of the present work, aimed at extracting physicochemical and molecular information from retention data, goes beyond the usual analytical or preparative applications of HPLC and falls in the domain of "molecular chromatography." The results obtained by the chromatographic approach with a set of dansylamino acids as model compounds in HIC at 25°C are in agreement with those of calorimetric studies on other processes governed by the hydrophobic effect: nonlinear van't Hoff plots, negative heat capacity, and mostly positive entropy changes. An important finding of this work is that the high-performance liquid chromatograph, a ubiquitous laboratory instrument, can be used for the measurement of data which previously required the use of a calorimeter.

It is intriguing to investigate whether the thermodynamic data obtained by molecular HIC confirms the existence of certain empirical relationships that have emerged from calorimetric studies on processes driven by hydrophobic interactions. Plots of enthalpy and entropy against heat capacity are linear at 25°C for processes involving hydrophobic interactions (26). Such relationships are expected to serve as diagnostic tools for mechanistic comparison of various processes. Detailed treatment of this subject will be presented in a subsequent paper.

The results presented here suggest that the same hermeneutics as that used for HIC can be applied to the retention in reversed-phase chromatography with water-rich mobile phases. Indeed, retention data with small peptides in reversed-phase chromatography has yielded similar results (41). In such investigations HPLC is expected to be not only a useful adjunct to calorimetry but a simple and convenient tool *per se* for the evaluation of thermodynamic parameters.

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