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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF SMALL MOLECULES: CHARACTERIZATION OF THE RETENTION OF ACYL COENZYME A HOMOLOGUES

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SUMMARY

The hydrophobic interaction chromatography (HIC) of C_2–C_{10} acyl coenzyme A (CoA) homologues was investigated in the isocratic mode. Retention of the homologues and of CoA fragments is well described by the empirical salting-out equation, $\log k' = \log k'_0 + mM_s$. Both slope and intercept in this equation increase with carbon number of the homologous series. Simple additivity with carbon number was not found for either slope or intercept. Each additional methylene group had a greater effect on increasing retention than the preceding methylene group. Comparison of the retention of structural fragments of CoA to that of acyl CoA homologues shows that the alkyl moiety of the homologues dominates their retention. The average free energy of transfer of a methylene group from water to a propyl HIC stationary phase was estimated as -280 cal/mol. The dependence of this free energy of transfer on salt concentration is found to be -50 cal/mol per 1 M increase in ammonium sulfate concentration.

INTRODUCTION

Small-molecule investigations in hydrophobic interaction chromatography (HIC) are uncommon since HIC is usually associated with protein separations. Although HIC has proven to be useful in separating proteins under stabilizing conditions, the retention mechanism(s) are not well understood. A study of the retention parameters of smaller molecules should provide a simpler interpretable system.

Although homologous series have been used extensively in reversed-phase chromatographic studies, there have been no reports of analogous studies in HIC. A homologous series of acyl coenzyme A (CoA) derivatives provides a useful means for probing the effect(s) of analyte structure on retention in HIC.

A linear increase has been reported in the log of the capacity factor (k') with increasing carbon number for several reversed-phase systems[1–4]. These observations have given rise to empirical models relating analyte structure to retention[2,5–9]. The idea that retention parameters of structural components of an analyte are additive in

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the retention of the entire analyte molecule is not new. The Martin rule of additivities of molecular increments[10,11] has been extensively applied to predict retention of an analyte based on its structural characteristics[12–14]. For example, quantitative structure–retention relationships (QSRR) based on this premise are widely applied in drug development as a means of characterizing molecular structure of analytes[15,16].

The present studies were carried out with a typical HIC stationary phase, using solvent compositions and operating conditions that are conventionally applied in HIC. Systematic capacity factor determinations are reported for several acyl CoA homologues as a function of ammonium sulfate concentration in the mobile phase. Additivity of the log of the capacity factor with increasing carbon number is not found for this series. The influence of ammonium sulfate concentration in the mobile phase on the free energy of transfer of a structural unit, the methylene group, to the stationary phase is discussed.

EXPERIMENTAL

Materials

Acetyl, *n*-propionyl, *n*-butyryl and *n*-hexanoyl CoA were obtained as their lithium salts from Sigma (St. Louis, MO, U.S.A.). *n*-Octanoyl and *n*-decanoyl CoA were obtained in the free acid form from Sigma and PL Biochemical (Milwaukee, WI, U.S.A.), respectively. CoA was obtained as its lithium salt from Pharmacia (Piscataway, NJ, U.S.A.). Adenosine and adenosine 5′-monophosphoric acid were obtained from Sigma. Ammonium sulfate (ultrapure grade) was obtained from Schwarz-Mann Biotech (Cleveland, OH, U.S.A.). All other reagents were of A.C.S. certified analytical-reagent grade.

Methods

The chromatographic system consisted of two Waters Model M6000A pumps, a Rheodyne Model 7125 injection valve, a 6.5-μm, 150 × 4.6 mm I.D. SynChropak propyl hydrophobic interaction column (SynChrom, Lafayette, IN, U.S.A.), and a Hewlett-Packard (Avondale, PA, U.S.A.) 3390A reporting integrator. The column dead time was determined by water injection.

Mobile phases were prepared with high-purity high-performance liquid chromatography (HPLC)-grade water obtained in-house with a Millipore (Bedford, MA, U.S.A.) Milli-Q water purification system as follows: mobile phase A: 2.1 *M* ammonium sulfate, 0.02 *M* potassium dihydrogen-phosphate, adjusted to pH 7.0 with a sodium hydroxide solution; mobile phase B: 0.02 *M* potassium dihydrogen phosphate, adjusted to pH 7.0 with a sodium hydroxide solution.

The composition of the mobile phase was controlled by a Waters Model 660 solvent programmer. Stock solutions of each CoA homologue were prepared by using high-purity HPLC-grade water (unbuffered) at a concentration *ca.* 0.2 mg/ml. Mobile phases and stock solutions were filtered through a Millipore HA (0.45 μm) filter and stored at 4°C when not in use. A 20-μl injection loop was used for all injections (approximately 4 μg per injection). The acyl CoA homologues were detected at 260 nm using a Waters Lambda-Max Model 480 spectrophotometer. The flow-rate was 1.0 ml/min throughout the study. The chromatographic column was maintained at 30 ± 0.2°C with a circulating-water jacket.

RESULTS AND DISCUSSION

It has been proposed that in HIC the log of the capacity factor, k', is linearly related to the surface tension (σ) of the mobile phase[17]

$$\log k' = A + B\sigma \tag{1}$$

and that surface tension is a linear function of salt concentration, M_s[18]

$$\sigma = \sigma_o + tM_s \tag{2}$$

Therefore, the relationship of log k' to ammonium sulfate concentration is given by eqn. 3

$$\log k' = \log k'_0 + mM_s \tag{3}$$

This equation is of the same form as the Setschenow equation for the salting out of non-polar compounds from aqueous solution[19], and presumably reflects a similar mechanism. The coefficient m is presumed to reflect the contact area between the analyte and the stationary phase.

For each of the six CoA derivatives, isocratic HIC data were acquired with mobile phases ranging in concentration from 2.1 to 0.0 M ammonium sulfate. A plot of eqn. 3 for each of the CoA derivatives is shown in Fig. 1.

Initially, the effect of added salt at low salt concentrations on ionized species is to reduce electrostatic interaction between the analytes and the stationary phase[20]. Retention of highly negatively charged molecules like acyl CoA homologues will probably involve substantial electrostatic interaction at very low salt concentrations.

Intercept (log k'_0) and slope (m) values for each acyl CoA homologue obtained from the lines of best fit of log k' *versus* ammonium sulfate concentration (eqn. 3) are plotted against carbon number of the homologue (Figs. 2 and 3). The values of log k' at

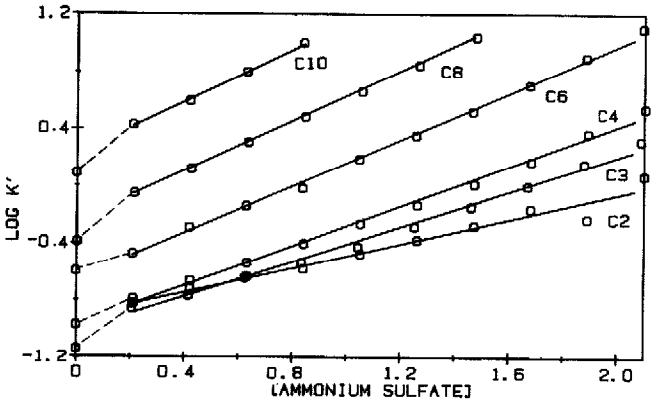

Fig. 1. Retention of acyl CoA derivatives on a SynChropak propyl column as a function of ammonium sulfate concentration. The carbon number of the acyl group identifies each curve. Each point represents the average of 3 or 4 t_R measurements.

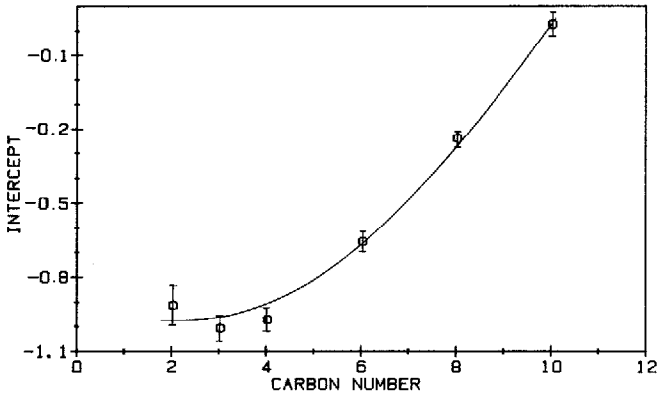

Fig. 2. Intercepts from log k' *versus* $[(NH_4)_2SO_4]$ plot, Fig. 1, as a function of carbon number in the acyl group. Error brackets indicate the 95% confidence interval, computed on the conservative premise that each point in Fig. 1 represents a single observation.

zero ammonium sulfate concentration were omitted from regression analysis because of the electrostatic considerations mentioned above. In recent versions of Sinanoglu's solvophobic theory[21], the slope (B) of eqn. 1 is taken to measure the change in solvent-exposed non-polar area on binding; the slope m eqn. 3 may be likewise identified. Fig. 2 and 3 show a kind of complementarity: slopes change most rapidly with carbon number in the low end of the range where intercept is statistically invariant, and intercepts change rapidly at the high end of the range where slope appears to approach a limiting value. Without providing any details, these results suggest some difference in the kind of interaction for the low carbon number and the high carbon number homologues with the stationary phase. The trends shown in Figs. 2 and 3 show that simple additivity is not found in slope or intercept.

Isocratic log k' values for each of the CoA derivatives were plotted *versus* carbon number (Fig. 4). Above carbon number 3 these plots yield approximately linear

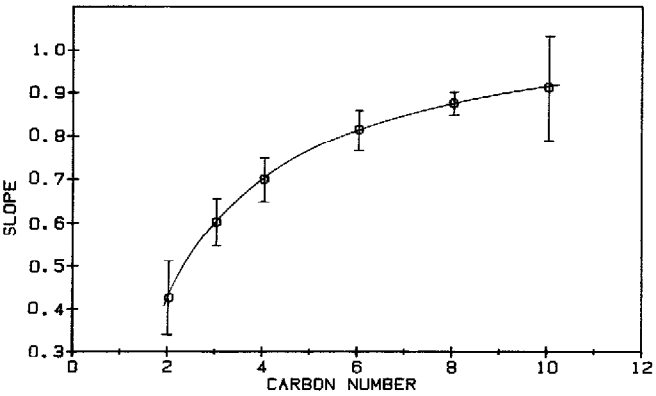

Fig. 3. Slopes from log k' *versus* $[(NH_4)_2SO_4]$ plot, Fig. 1, as a function of carbon number in the acyl group. Error brackets indicate the 95% confidence interval as in Fig. 2.

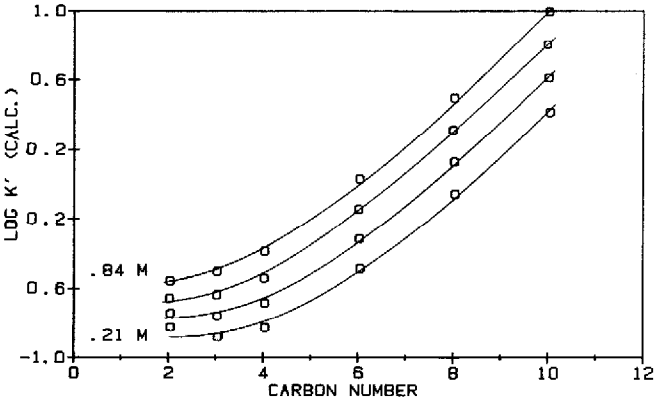

Fig. 4. Variation of k' (data from Fig. 1 smoothed by linear regression) with carbon number, at four salt concentrations. The successive curves, from the bottom up, represent isocratic data at 0.21, 0.42, 0.63, and 0.84 M $(NH_4)_2SO_4$, respectively.

relationships (Fig. 4, $r > 0.997$ for all plots, $n = 4$). A linear dependence of the log of the capacity factor on the number of repeating molecular units in a homologous series is well established in reversed-phase chromatography[1,22,23].

Closer inspection of Fig. 4, however, reveals that the log k' values (including carbon numbers 2–4) are better related to carbon number by a second order polynomial. The first few members of a homologous series may not contribute equally to retention because of end effects caused by the spatial proximity of the first few methylene groups to the thioester moiety of the homolog[1]. Other examples of a non-linear relationship between retention and carbon number have been reported[15,24,25]. Studies in reversed-phase chromatography by Jandera[26] have shown that while linear fits are often quite good for a homologous series, more generality is achieved with a quadratic formulation. He derived the following equation relating log k' to carbon number from interaction index theory[9]:

$$\log k' = \log \beta + (\log \alpha) n_c + (\log \gamma) n_c^2 \quad (4)$$

The term α represents the retention ratio between two adjacent members of a homologous series. In a reversed-phase study of a homologous series, Colin *et al.*[5] defined log α as "solvophobicity" and found that this parameter varied almost linearly with the water content in a methanol–water mobile phase system. Analogously in this hydrophobic interaction study, "solvophobicity" is related to the salt concentration in the mobile phase. The intercept, log β, is a measure of the specific selectivity in a homologous series[26] and represents the interaction between the acyl CoA molecule ($n_c = 0$) with the stationary phase. The coefficient of the squared term, log γ, is usually insignificant enough to ignore in reversed-phase systems so that log k' is related to carbon number by the linear relationship[21]

$$\log k' = \log \beta + (\log \alpha) n_c \quad (5)$$

The curvature in the plot of log k' *versus* carbon number (Fig. 4) indicates that the slope (log α), which is a measure of methylene selectivity, increases with the carbon number of the series. Also, as the salt concentration in the mobile phase increases, log β increases (Table I), reflecting the increased retention contributed by the acyl CoA moiety ($n_c = 0$) at higher salt concentrations. It is evident from Fig. 4 that in HIC (for this homologous series) the coefficient of the squared term (log γ) of the empirically derived equation is not insignificant.

The relative contribution to retention of the CoA moiety of the homologues was assumed to be constant at a particular mobile phase composition. This assumption has been applied for molecules larger than the *n*-alkanes commonly used in a homologous series study. For example, cholesterol homologues have been extensively treated in this way in reversed-phase chromatography[2]. However, an analyte may be bound to the stationary phase in several orientations, each with a characteristic affinity. In a homologous series the distribution of binding orientations may change from one homologue to the next. This would result in the same fragment of the analyte contributing different amounts of binding energy in different homologues. Such effects may be responsible for the failure of strict additivity in slope and intercept along the homologous series (Figs. 2 and 3), as noted above.

Retention of structural fragments of CoA

Plots of log k' *versus* ammonium sulfate concentration for adenosine and adenosine 5′-monophosphate (AMP) are shown in Fig. 5 along with plots for CoA, propionyl and hexanoyl CoA. The experimental conditions are the same as for Fig. 1, except that a different lot of SynChropak propyl column packing was used. The line of best fit for hexanoyl CoA plot has a significantly greater slope and intercept than propionyl CoA, similar to the plots of Fig. 1. The line of best fit for CoA has a smaller slope and intercept than that of propionyl CoA, but the difference is markedly less than the same three-carbon compositional difference between propionyl and hexanoyl CoA. It is evident that retention for this homologous series is strongly influenced by the length of the alkyl chain. While the structural fragments of the CoA molecule represented by adenosine and AMP obviously contribute to retention of the acyl CoA homologues, their effect on retention is outweighed by that of the alkyl chain of the homologue as the carbon number of the chain increases.

Further inspection of Fig. 5 shows nearly the same slopes for adenosine and

TABLE I

SECOND ORDER POLYNOMIAL FITS OF LOG k' *versus* CARBON NUMBER, FOR SEVERAL $(NH_4)_2SO_4$ CONCENTRATIONS (M_s)

$\log k' = \log \beta + (\log \alpha) n_c + (\log \gamma)\, n_c^2$.

$(NH_4)_2SO_4$ M_s	1st term log β	2nd term log α	3rd term log γ	Correlation coefficient (r)
0.210	−0.84326	−0.054278	−0.018336	−0.9957
0.420	−0.81104	−0.018747	−0.016349	−0.9969
0.630	−0.77718	−0.016249	−0.014392	−0.9978
0.840	−0.74340	−0.051088	−0.012462	−0.9985

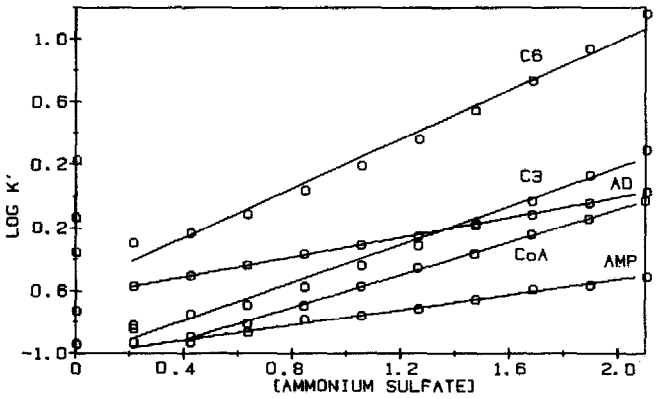

Fig. 5. Retention of various structural components of acyl CoA as a function of ammonium sulfate concentration. AD = Adenosine; AMP = adenosine 5'-monophosphate; CoA = coenzyme A, C_3 = propionyl CoA; C_6 = hexanoyl CoA. Experimental conditions are the same as Fig. 1, except that a different lot of SynChropak propyl column packing was used.

AMP, but a substantially lower intercept for AMP. In terms of the simple interpretation of eqn. 3 (above), the contact areas are nearly the same for the two compounds, and introduction of the hydrophilic phosphate ester into adenosine adds a repulsive component to the attractive hydrophobic interaction. Comparison of the retention curves of adenosine and CoA shows that while the slope for CoA is greater than for adenosine, CoA is less strongly bound over the range of salt concentration examined. Interpreting the slope of eqn. 3 as before, this result means that while CoA exhibits a greater contact area than adenosine, that additional contact area (the phosphopantetheine moiety) contributes a repulsive component to the hydrophobic binding.

Free-energy considerations

Since the log of the capacity factor is related to ΔG by the well-known equation

$$\log k' = \log \varphi - \Delta G/2.3\ RT \tag{6}$$

where φ is the phase ratio and is assumed to be constant, the free energy of transfer of a methylene moiety, ΔG_{CH_2}, can be calculated. From our data, ΔG_{CH_2} can be estimated from the slope of a linear fit of log k' *versus* carbon number[27], by using only log k' values for homologues of $n_c > 3$.

$$\Delta G_{CH_2} = -2.303\ RT\ (\Delta \log k'/n_c) \tag{7}$$

The slopes of log k' *versus* carbon number (Fig. 4, n_c = 4, 6, 8, 10) and the estimated free energies of transfer of a methylene group from the mobile phase to the stationary phase are given in Table II.

Each free energy listed in Table II is the free-energy contribution of a methylene group approximated from a linear fit. Confidence intervals are not reported because the calculation of the errors in the slopes determined by a linear fit are valid only if the

TABLE II
FREE ENERGY OF BINDING IN HIC, PER CH_2 GROUP

$(NH_4)_2SO_4$	Slope ($\Delta \log k'/n_c$)	ΔG_{CH_2} (cal/mol)
0.000[a]	0.1694[a]	−235[a]
0.210	0.2081	−288
0.420	0.2154	−299
0.630	0.2227	−309
0.840	0.2300	−319
0.000[b]	—	−278[b]

[a] These values were not included in determining the slope of Fig. 6 (see text).
[b] This pair of values was obtained from extrapolation of the data of Fig. 6.

data are linear, and clearly, the data are better fit to a quadratic equation for each isocratic mobile phase. Each additional methylene moiety contributes a little more to retention than the previous methylene group. Therefore, only an "average" free-energy contribution is estimated.

Since the slope values listed in Table II are linearly related to the concentration of ammonium sulfate in the mobile phase, it follows that the free energy of transfer of a methylene moiety is linearly related to salt concentration. A plot of the free energies (Table II) *versus* ammonium sulfate concentration (M_s) yields a slope of approximately -48.2 ± 0.7 (95% confidence interval) cal/mol per 1 M ammonium sulfate increase (Fig. 6). Because of probable electrostatic contributions, 0.0 M ammonium sulfate data are not included in this linear fit.

It is of interest to compare the experimentally derived free energy of transfer values in this study to values derived from liquid–liquid partition and reversed-phase chromatography experiments. Tomlinson *et al.*[24] have shown that a comparison of liquid–liquid partition to reversed-phase chromatography retention data is thermodynamically valid. For example, Zaslavsky *et al.*[28], calculated the free energy of transfer of the methylene moiety from water to hexane (using data acquired by Fendler *et al.*[29]) to be −692 cal/mol at 25°C. By reversed-phase chromatographic means,

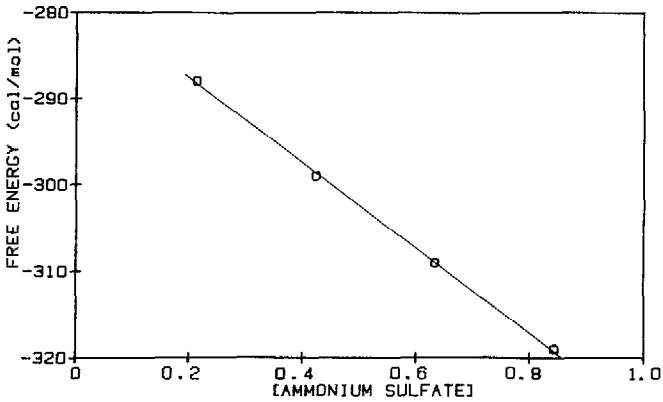

Fig. 6. "Average" free energy of transfer of a methylene group (range C_4 to C_{10}) from mobile to stationary phase, as a function of $[(NH_4)_2SO_4]$. Data from Table II.

Tanaka and Thornton[30] studied the partitioning of homologous series of alkanes and long-chain carboxylic acids between water and reversed-phase (C_{18}) chromatographic stationary phases and calculated a ΔG_{CH_2} on order of -700 cal/mol at 30°C.

The free-energy comparison given here is not meant to be used as a direct comparison of a C_3 hydrophobic interaction column with a C_{18} reversed-phase column, since a C_3 column has a lower percentage carbon loading (lower density, short alkyl chains) than a reversed-phase column (higher density, long alkyl chains). However, our calculation of $\Delta G_{CH_2} = -280$ cal/mol (at 30°C) does suggest that the alkyl moieties of the CoA derivatives are substantially less solvated by the hydrocarbon moieties on the C_3 stationary phase employed in our study than by the methylene group's partition from water into hexane ($\Delta G_{CH_2} = -692$ cal/mol at 25°C[28]) or partition from water into a C_{18} stationary phase ($\Delta G_{CH_2} = -700$ cal/mol at 30°C[30]). We suggest that geometric constraints must preclude total immersion of alkyl groups of the analyte in the thin and non-uniform layer of propyl moieties of the polymeric HIC stationary phase.

Stationary phase contributions

A relative change in selectivity of 11% resulted in our hydrophobic interaction study (Table I) within a change in mobile phase composition of approximately 0.6 *M* ammonium sulfate (the stationary phase remaining constant). The magnitude of this change in selectivity on the propyl HIC column (due to less than a 1 *M* change in salt concentration) can be better appreciated when realizing that a change in a reversed-phase column from C_8 to C_{18} would result analogously in a 10% relative selectivity change (since it is commonly accepted for the highly carbon-loaded reversed-phase column that there is an approximately 1% increase in selectivity per carbon atom[8]). The magnitude of the relative selectivity change in this HIC study is not surprising and supports the premise that the influence of the stationary phase on retention in HIC is outweighed by the influence of the mobile phase. This premise is generally accepted in RPC[23].

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