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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF ALIPHATIC ALCOHOLS AND CARBOXYLIC ACIDS ON OCTYL-SEPHAROSE CL-4B: MECHANISM AND THERMODYNAMICS

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SUMMARY

The retention of homologous aliphatic alcohols and carboxylic acids in dilute phosphate buffer solution was measured on octyl-Sepharose CL-4B and on unsubstituted Sepharose CL-4B as a function of chain length, pH and temperature. From the retention data the standard thermodynamic functions (ΔG° , Δc_p° , ΔH° , ΔS°) of the interaction were calculated. The thermodynamics and mechanism of the retention process are discussed.

INTRODUCTION

Hydrophobic interaction chromatography is a relatively new separation method that is rapidly finding applications in biochemistry. As it exploits a parameter, *i.e.* hydrophobicity, that is not directly used in the common separation methods based on differences in electrical and size parameters, it is a welcome additional tool in the biochemist's laboratory. With the introduction of uncharged alkyl-agarose gels, separations based on differences in hydrophobicity, free from interferences from charge and exclusion effects, have been made possible.

The classical explanation of the interaction of non-polar moieties in aqueous solution, as put forward by Frank and Evans¹, Kauzmann² and others, is that of an essentially entropy-driven hydrophobic interaction. In this view, the interaction between the non-polar moiety of the solute and that of the chromatographic support is mainly the result of the decrease in order when water molecules of the relatively ordered hydration structures (the so-called 'icebergs') surrounding the non-polar groups are released to the less-ordered bulk water. This gives rise to an entropy increase that is supposed to outweigh the accompanying enthalpy change: $\Delta H \ll T\Delta S$. A small value of the enthalpy change is conceived, as the energy needed for the disruption of hydrogen bonds in the 'iceberg' structure is of the same order of magnitude as the energy released in the formation of new hydrogen bonds between water molecules refilling the cage left by the non-polar moiety.

In the hydrophobic interaction chromatography of proteins, adsorption is gen-

erally obtained by using high salt concentrations in the aqueous adsorption buffer and desorption is brought about either by decreasing the salt concentration in the elution buffer (or by replacing the salt by a 'chaotropic' one) or by adding a less polar cosolvent to the eluent. However, these changes, apart from influencing the hydrophobic interaction, might also affect the intrinsic structural properties of the proteins and, perhaps, of the adsorbent. Therefore, it is difficult to interpret the results of protein chromatography in terms of hydrophobic interactions *per se*. Thus, to the authors' knowledge, only one paper³ has been published in which the relation $\Delta H \ll T\Delta S$ was shown to prevail for protein adsorption from aqueous solution to (charged) alkyl-agarose.

In this paper we present results of chromatographic experiments with homologous aliphatic monoalcohols and -carboxylic acids on octyl-Sepharose CL-4B. The experiments were performed at several temperatures between 4 and 40°C and, for the alcohols, at widely different pH values, enabling us to determine separately the influence of pH and the nature of the polar head groups on the thermodynamics of the interaction of the hydrocarbon tail with octyl groups on the chromatographic support.

Whereas the use of these small test compounds circumvents interpretation difficulties as their structure is invariable, it should be emphasized that the interaction of proteins with the chromatographic adsorbent is much more complicated than that of our simple test compounds. For example, in the interaction of a glutamic acid side-chain with octyl-agarose, the terminal carboxylic group almost certainly has to leave the aqueous eluent, whereas butyric acid (one of our test compounds) is in principle capable of hydrophobic interactions whilst keeping its polar head in the eluent. One should realize this if one wishes to extend the conclusions arrived at in this paper to the hydrophobic interaction chromatography of proteins.

THEORETICAL

The retention on octyl-Sepharose of small amphiphilic compounds with a relatively large non-polar group can be expected to result mainly from hydrophobic interactions. However, other interactions could also play a role. First, electrostatic interactions could occur between residual charged groups on the matrix and charged amphiphiles; for the uncharged test compounds studied in this paper such interactions are non-existent. Second, hydrogen bonds could be formed between the head groups of our test compounds and certain functional groups on the agarose matrix, *e.g.*, between OH or COOH head groups and agarose hydroxyls or agarose ether oxygens. The magnitude of this type of interaction can be established experimentally (and its contribution to the retention on octyl-Sepharose subsequently corrected for) by measuring the chromatographic retention on unsubstituted Sepharose under identical conditions.

Such a correction is, of course, not adequate if hydrophobic and hydrogen bonding interactions are cooperative. This also holds for the contribution due to hydrogen bonding of the head groups of our test compounds with hydroxyl and ether oxygen in the spacer arm connecting the octyl group to the agarose matrix. This means that the results on individual test compounds need careful interpretation. However, if we compare the results obtained for members of a homologous series,

i.e., focus on the interpretation of methylene increment values, the above effects can be expected to cancel.

The most appropriate model for the retention of small amphiphilic compounds on octyl-agarose depends on the density of octyl groups on the gel matrix. If the octyl groups are far apart (*i.e.*, at an average distance greater than the length of the alkyl chain of the solute) it is evident that the retention of the solute should be described as a result of a bimolecular association of the solute with single octyl groups on the gel. A correction should then be applied for retention caused by possible interaction of the solute with the agarose constituent of the matrix. (Interaction of small amphiphiles, *e.g.*, aliphatic monoalcohols, with highly cross-linked Sephadex gels is a well documented phenomenon⁴.) If, on the other hand, the octyl group density is high, such a description is no longer adequate, as the solute can interact simultaneously with more than one octyl group. At extremely high octyl group densities it is even conceivable that the agarose chains of the matrix will be fully covered by an octyl layer, preventing the solute from interacting with agarose. In such an extreme situation, the retention of the solute can be described as a result of its 'partition' between the mobile phase and the octyl layer on the gel. (Still another picture of the retention mechanism is adsorption of the alkyl chain of the solute on an octyl layer surrounding the agarose matrix. Interpretation in terms of this mechanism is hampered, however, by the lack of information on the octyl surface area.)

Unfortunately, the octyl group density on octyl-Sepharose CL-4B is not known with certainty. From the proposed double helix structure of agarose⁵ and the known octyl content of the gel⁶ (about 0.2 mole of octyl per mole of galactose) a mean octyl group density on an agarose double helix of $5 \cdot 10^{-3} \text{ \AA}^{-2}$ can be calculated⁷. However, in Sepharose 4B, the parent matrix of octyl-Sepharose CL-4B, several agarose double helices are aggregated into fibres. This probably also holds for octyl-Sepharose CL-4B, as neither cross-linking⁸ nor the introduction of octyl groups⁹ seems to alter the permeability of the gel substantially. Obviously, such an aggregation would lead to a greater octyl group density than that indicated above. Unfortunately, the degree of aggregation is uncertain. On the one hand it was estimated¹⁰ from electron microscopy of Sepharose 4B to range from one to several hundred double helices per fibre; on the other, application of the treatment of Laurent and Killander¹¹ to the retention data of Ficoll fractions on 4% agarose yielded¹² a mean fibre radius of 25 Å, corresponding to an aggregation of about ten double helices per fibre. In a recent electron microscopy study¹³ the degree of aggregation was estimated to be 10–30 double helices per fibre. Based on a mean value of 20, about a 4.5 times higher mean octyl group density (*i.e.*, $23 \cdot 10^{-3} \text{ \AA}^{-2}$) would be expected for octyl-Sepharose CL-4B, corresponding to a mean distance of the points of attachment to the matrix of about 7 Å. This distance must be compared with the length of the alkyl groups of the amphiphiles used in this study, *viz.*, 2.5–8.5 Å.

Hence the available information on the octyl group density does not clearly point to which retention model should be used. However, experimental evidence, *i.e.*, (i) retention as a function of the octyl group density and (ii) the value of the CH₂ increment of the standard free energy of sorption, demonstrates that a simple bimolecular association does not occur on octyl-Sepharose CL-4B (see Discussion). We therefore used a partition model for the description of the retention data. The only complication with this model is that the weight of the octyl layer on the gel is

known, but not its density and hence not its volume. This makes it necessary to use molalities as concentration units.

The partition of a solute X between the aqueous mobile phase M and the stationary phase S (an octyl layer surrounding the agarose fibres) is described by the partition constant K_m :

$$K_m = \frac{(m_X)_S}{(m_X)_M} \quad (1)$$

where m_X represents the molality of X.

The elution volume V_e of the solute X on a column (bed volume V_{bed}) can be written as

$$V_e = V_M \left(1 + K_m \cdot \frac{W_S}{W_M} \right) \quad (2)$$

where V_M is the volume of the mobile phase in the column and W_S and W_M represent the weight of stationary and mobile phase, respectively. As $W_M = \rho V_M$, where ρ is the specific gravity of the mobile phase, eqn. 2 can be rearranged to

$$\frac{K_m}{\rho} = \frac{V_e - V_M}{W_S} \quad (3)$$

V_M can be evaluated if the specific bed weight, β_{os} , of octyl-Sepharose CL-4B (milligrams of matrix material per millilitre of gel bed) and the density, ρ_{os} , of the gel are known:

$$V_M = V_{bed} \left(1 - 10^{-3} \cdot \frac{\beta_{os}}{\rho_{os}} \right) \quad (4)$$

W_S is taken as the weight of octyl groups plus spacer arms through which they are attached to the agarose chains:

$$W_S = \frac{2pM_o}{M_{ru} + 2pM_o} \cdot V_{bed} \cdot 10^{-3} \beta_{os} \quad (5)$$

where p denotes the octyl concentration (moles of octyl per mole of galactose), M_o represents the molecular weight of the octyl group + spacer arm ($M_o = 186$) and M_{ru} that of the galactose-anhydrogalactose repeating unit in agarose ($M_{ru} = 306$). Note that the weight of the cross-links has been neglected in eqn. 5.

In deriving eqn. 3, it is tacitly assumed that the solute X is not involved in association processes, *e.g.*, micellization, in the mobile phase. The following arguments can be raised in favour of this assumption.

(i) The overall octyl concentration in the gel bed is about 400 mM, and that of the test compounds in the eluent is at maximum equal to the sample concentration, which ranged between 8 and 17 mM (see Experimental). It is difficult to imagine why,

under these circumstances, a solute molecule would 'prefer' self-association to association with octyl groups of the gel.

(ii) The elution peaks were generally symmetrical (see Experimental), which means that the retention is independent of the solute concentration. This fact cannot be reconciled with monomer-dimer (or micellar) equilibria in the mobile phase.

(iii) For the highest members of a homologous series the elution peaks were slightly asymmetric. If this were due to self-association in the mobile phase, *i.e.*, to a process reversed to the chromatographic retention, one would expect the free energy change per CH_2 group to diminish for these higher members. Such an effect is not observed, however (see Fig. 2).

EXPERIMENTAL

Octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was washed several times with the eluent and, after deaeration of the suspension, packed in a thermostatically controlled column (K16/70, Pharmacia) to a bed height of about 60 cm (bed volume about 120 ml) at an eluent flow-rate of about 15 ml h^{-1} . The column was equipped with a sample valve (LV4, Pharmacia), a flow adaptor (A16, Pharmacia), a peristaltic pump (Minipuls 2; Gilson, Villiers-le-Bel, France) and a $70\text{-}\mu\text{l}$ UV monitor (Uvicord S; LKB, Bromma, Sweden) or a $10\text{-}\mu\text{l}$ refractive index (RI) detector (R-403; Waters Assoc., Milford, MA, U.S.A.).

Sample solutions were prepared by dissolving the test compounds in the eluent to a concentration of about $1 \mu\text{l ml}^{-1}$ or 1 mg ml^{-1} . When (with some of the higher homologues) these concentrations could not be obtained, saturated solutions of the test compounds were used.

Aliquots of 1–2 ml were applied to the column and eluted at a flow-rate of about 15 ml h^{-1} . The purity of the test compounds (of various origins) was always $\geq 98\%$; they were used without further purification. Analytical-reagent grade compounds (Baker, Deventer, The Netherlands) and doubly distilled water were used to prepare the eluents. The eluent composition was 25 mM sodium phosphate, pH 3.52 (alcohols and carboxylic acids) or pH 7.10 (alcohols). At pH 3.52 the carboxylic acids are essentially uncharged. Chromatograms were obtained by recording the UV absorbance at 206 nm (carboxylic acids) or the RI signal (alcohols). Flow-rates were determined gravimetrically.

A few experiments with both series of test compounds were performed at different eluent flow-rates (10 and 30 ml h^{-1}). K values calculated from these experiments with eqn. 3, taking into account the slightly different specific bed weights, were identical within experimental error.

Some experiments were performed with hexanol at different solute concentrations (0.2 and 0.5 mg ml^{-1}); the elution volumes were identical with that at 1 mg ml^{-1} .

The elution peaks were generally symmetrical; the HETP values ranged from 0.03 to 0.06 cm. Some minor tailing was observed, however, with the highest members of a homologous series.

After completion of a series of measurements with a given eluent at a given temperature, about 10 ml of the gel bed on top of the column were slurried into the supernatant eluent and most of it was withdrawn. Subsequently the remaining slurry

was repacked at the same flow-rate, restoring a flat bed surface. The decrease in bed volume (ΔV) was measured. The gel slurry was transferred to a weighed glass filter, thoroughly washed with distilled water and ethanol-water mixtures of increasing ethanol content and dried at 90°C to constant weight (W). From these data the specific bed weight β_{os} (milligrams of octyl-Sepharose per millilitre of gel bed), pertaining to a given eluent and temperature, was calculated as $\beta_{os} = W/\Delta V$.

Octyl-agarose of varying degrees of octyl substitution was prepared from Sepharose CL-4B using the method of Hjertén *et al.*¹⁴. Its octyl concentration was determined by NMR spectroscopy¹⁵. Some of the test compounds were chromatographed on this material (at slightly different buffer concentrations and pH values).

RESULTS

In Fig. 1 K_m values, calculated according to eqns. 3–5, are given for the retention of hexanoic and heptanoic acids on octyl-agaroses of varying degree of octyl substitution.

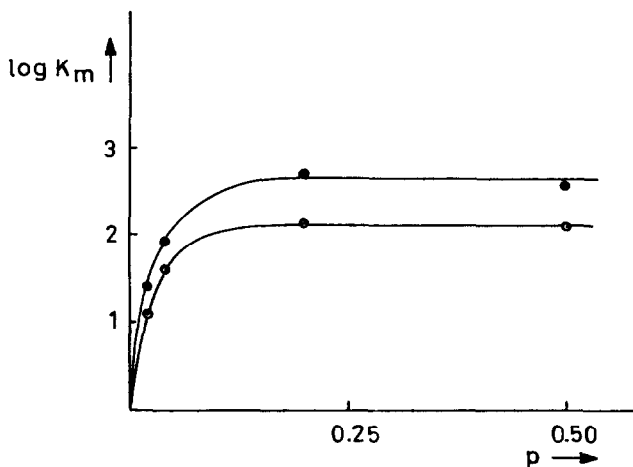


Fig. 1. K_m values at 25°C for hexanoic (O) and heptanoic acid (●) versus the degree of octyl substitution in octyl-agarose. Eluent: 5 mM phosphate, pH 2.7.

In Table I the elution volumes (corrected for dead volume and normalized to a bed volume of 100 ml) of the test compounds on octyl-Sepharose CL-4B and Sepharose CL-4B are given at different pH values of the eluent and at different temperatures. From the speed stability of the peristaltic pump and the time interval of eluent weighings relative to the retention times found, and also from the differences between occasional duplicates, we estimate the precision of these normalized values to be about 0.2%.

In Table II the specific bed weights of octyl-Sepharose CL-4B and Sepharose CL-4B are given at the same pH values and temperatures. The precision of these values is about 1%.

The data in Tables I and II were used to calculate K_m values using eqns. 3–5.

TABLE I

ELUTION VOLUMES (ml) OF THE TEST COMPOUNDS ON 100-ml BEDS OF OCTYL-SEPHAROSE CL-4B AND SEPHAROSE CL-4B

Eluent	Test compound	n	Octyl-Sepharose						Sepharose, 25°C*
			4°C	10°C	17.5°C	25°C	32.5°C	40°C	
25 mM sodium phosphate (pH 3.52)	C _n H _{2n+1} COOH	2	99.1	99.7	99.6	99.2	99.5	99.2	98.2
		3	102.5	103.5	101.9	101.7	101.2	101.3	98.3
		4	110.9	111.6	111.2	110.0	111.0	109.7	98.5
		5	143.2	144.3	142.7	140.8	139.1	135.6	98.4
		6	261.7	264.3	259.0	253.8	241.6	228.0	99.4
25 mM sodium phosphate (pH 3.52)	C _n H _{2n+1} OH	3	98.6	99.0	98.8	98.8	99.7	98.8	98.1
		4	99.9	100.8	100.5	100.7	100.7	101.6	98.1
		5	105.0	106.3	106.6	108.0	108.3	108.0	98.5
		6	124.6	127.1	129.8	133.3	134.1	133.3	98.8
		7	194.3	206.5	217.1	224.9	228.8	219.0	99.0
25 mM sodium phosphate (pH 7.10)	C _n H _{2n+1} OH	3	98.5	98.6	98.9	98.8	98.9	98.6	97.7
		4	100.8	101.3	101.1	102.3	102.2	102.7	97.8
		5	110.6	111.3	112.8	113.9	115.1	115.8	98.0
		6	143.3	147.9	153.9	156.4	160.7	161.7	97.9
		7	270.0	288.2	306.1	316.1	328.8	327.8	98.1

* Elution volumes on Sepharose CL-4B were independent of temperature (within experimental error).

TABLE II

SPECIFIC BED WEIGHT (mg/ml) OF OCTYL-SEPHAROSE CL-4B

Eluent	Temperature (°C)					
	4	10	17.5	25	32.5	40
25 mM sodium phosphate (pH 3.52)	35.1	34.3	33.1	31.7	30.0	28.1
25 mM sodium phosphate (pH 7.10)	37.3	37.1	36.3	36.3	35.4	33.4

In this calculation we assumed $\rho_{os} = 1.6$ (approximately the density of a related polysaccharide, Sephadex G-200). The octyl concentrations, p , for the batches of octyl-Sepharose CL-4B used were given by the manufacturer.

Values of the (partial molal) standard free energy change for the partition process (ΔG°) were determined from the equation

$$\Delta G^\circ = -RT \ln K_m \quad (6)$$

In Fig. 2 ΔG° values at 25°C are plotted against the chain length, n , for the three series of measurements.

Fig. 3 shows a typical Van 't Hoff plot ($\Delta G^\circ/T$ versus $1/T$) for one of the series, the alcohols at pH 7.10.

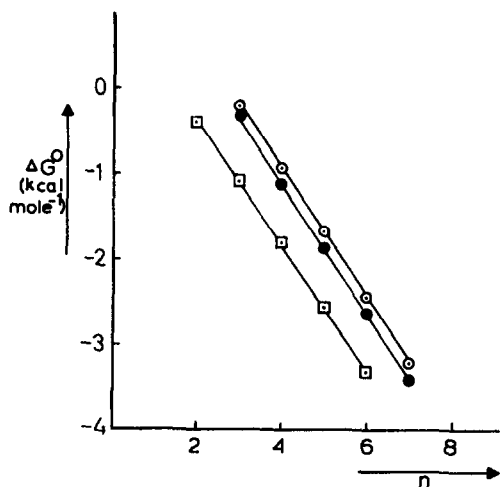


Fig. 2. Standard free energy at 25°C versus chain length for carboxylic acids at pH 3.52 (\square) and alcohols at pH 3.52 (\circ) and pH 7.10 (\bullet).

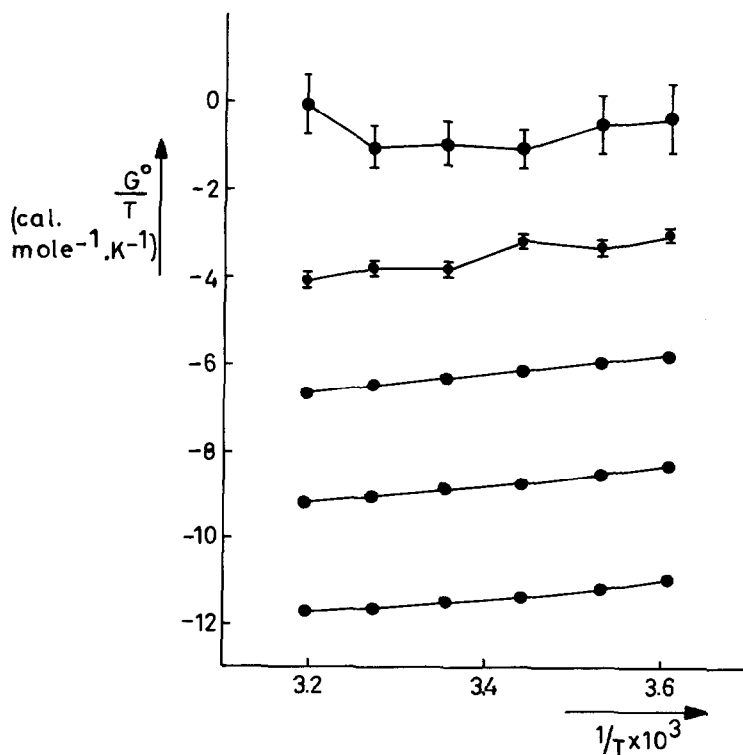


Fig. 3. Van't Hoff plot for alcohols in 25 mM phosphate buffer at pH 7.10.

Values of the standard thermodynamic quantities ΔC_p° , ΔH° and ΔS° at 25°C were calculated with eqn. 7. In this equation, ΔC_p° is assumed¹⁶ to be independent of temperature and $\theta = 298.1^\circ\text{K}$.

$$Y \equiv \frac{\Delta G^\circ}{T} - \Delta C_p^\circ \left(1 - \frac{\theta}{T} - \ln \frac{T}{\theta}\right) = -\Delta S_\theta^\circ + \Delta H_\theta^\circ \cdot \frac{1}{T} \quad (7)$$

For this purpose, values of Y were calculated with different pre-set ΔC_p° values. Sets of Y versus $1/T$ data were analysed with the least-squares treatment for linear regression. The values of ΔC_p° that gave the best fit to the linear equation $Y = a + b \left(\frac{1}{T}\right)$ were retained. The slope b and the intercept a of this line were equated to ΔH_θ° and ΔS_θ° , respectively. The results are given in Table III and Fig. 4. Note that this treatment is unable to extract thermodynamic data from the results for the first members of each series in Table I, as a result of the large experimental errors involved.

TABLE III
THERMODYNAMIC DATA FOR BINDING TO OCTYL-SEPHAROSE CL-4B

ΔC_p° , ΔS° and s_y in cal °K⁻¹ mole⁻¹; ΔH° in cal mole⁻¹

Eluent	pH	Test compound	<i>n</i>	ΔC_p°	ΔH°	ΔS°	s_y
25 mM sodium phosphate	3.52	C _n H _{2n+1} COOH	3	35	-1589 ± 657	-1.6 ± 2.2	0.23
			4	-13	291 ± 265	7.2 ± 0.9	0.09
			5	-39	-65 ± 61	8.4 ± 0.2	0.02
			6	-61	-330 ± 31	10.0 ± 0.1	0.01
25 mM sodium phosphate	3.52	C _n H _{2n+1} OH	4	48	2569 ± 764	11.7 ± 2.6	0.27
			5	-83	2205 ± 186	13.0 ± 0.6	0.07
			6	-65	2182 ± 91	15.5 ± 0.3	0.03
			7	-113	1766 ± 85	16.7 ± 0.3	0.03
25 mM sodium phosphate	7.10	C _n H _{2n+1} OH	4	32	2592 ± 482	12.3 ± 1.6	0.17
			5	11	2127 ± 33	13.4 ± 0.1	0.01
			6	-30	1949 ± 77	15.4 ± 0.3	0.03
			7	-40	1691 ± 55	17.2 ± 0.2	0.02

From the precision of the data in Tables I and II, the expected error in $\Delta G^\circ/T$ can be estimated. In Fig. 5 this error is shown in a logarithmic plot as a function of the elution volume V_e . It can be seen that for $V_e > 150$ ml the error is essentially constant as it is governed by the precision of the specific bed weight data (Table II). For $V_e < 120$ ml it increases steeply with decreasing V_e as the difference $V_e - V_M$ (eqn. 3) becomes increasingly small.

For comparison, the error s_y in a single Y -value (Table III), as found in the least-squares treatment, is also indicated in Fig. 5, for each test compound. It can be inferred that in general the consistency of the data at different temperatures is satisfactory: only in a few cases is s_y larger than expected from measurements at a single temperature.

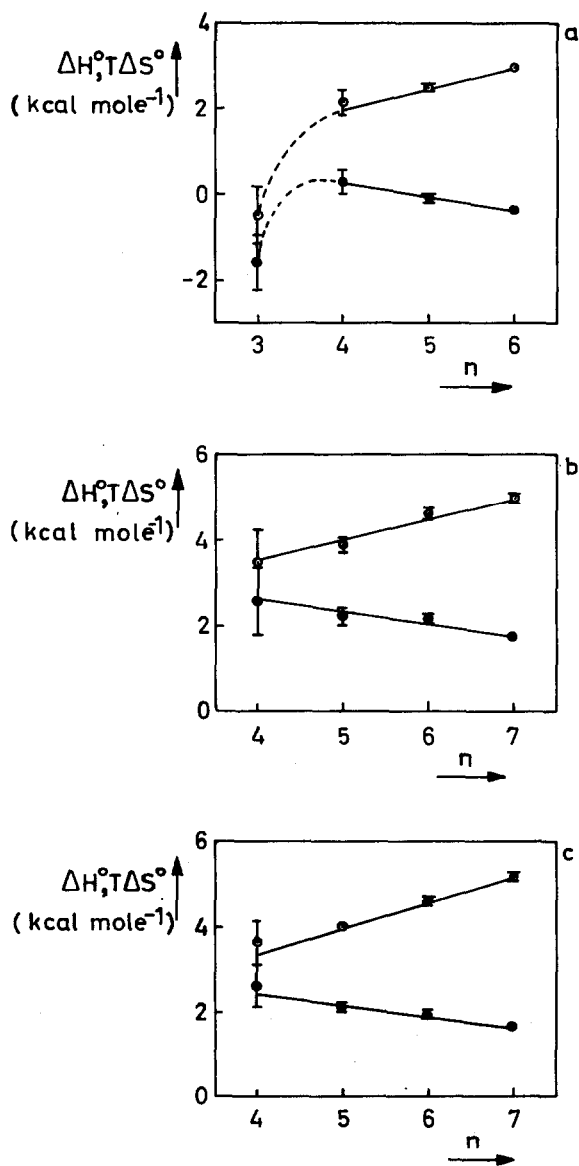


Fig. 4. Values of ΔH° (●) and $T\Delta S^\circ$ (○) at 25°C versus chain length. (a) Carboxylic acids (pH 3.52); (b) alcohols (pH 3.52); (c) alcohols (pH 7.10).

DISCUSSION AND CONCLUSIONS

Retention on unsubstituted Sepharose

The results in the last column of Table I give rise to the following remarks. From the specific bed weight of Sepharose CL-4B (Table II) and an assumed density of the polymer of 1.6 g ml $^{-1}$, a total volume of mobile and stationary phase in a

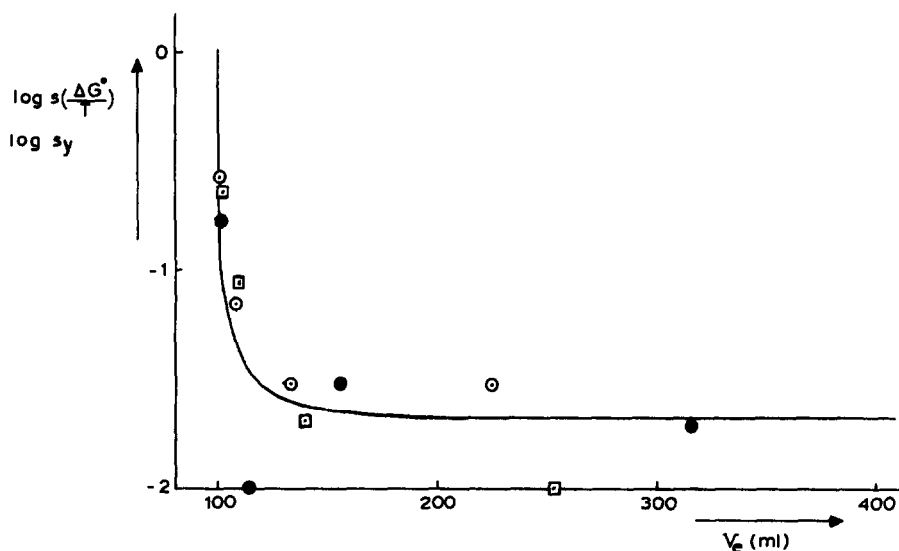


Fig. 5. Calculated standard error in $\Delta G^\circ/T$ (—) and experimental standard errors in Y versus elution volume on a 100-ml octyl-Sepharose column at 25°C. Notation of experimental data as in Fig. 2.

100-ml bed of 97.9 and 97.8 ml can be estimated in 25 mM buffer at pH 3.52 and 7.10, respectively.

At pH 3.52, the elution volumes of alcohols and carboxylic acids are greater than 97.9 ml and increase with increasing chain length. This indicates a small but measurable interaction of the non-polar part of the amphiphiles with the polysaccharide. Such an interaction, but more pronounced, was also found on highly cross-linked Sephadex by Haglund and Marsden⁴.

It is not clear why the same effect is not found with the alcohol series at pH 7.10, especially as the interaction of alcohols with octyl-substituted Sepharose is stronger at pH 7.10 than at pH 3.52. A possible reason could be that at pH 7.10 the matrix contains some residual charged carboxylate groups, preventing hydrophobic interaction of the alcohols with the matrix (but not with octyl groups on the octyl-substituted matrix).

For all but the lowest members of each homologous series the retention on unsubstituted Sepharose is small compared with that on octyl-Sepharose. Therefore, elution volumes on octyl-Sepharose were not corrected for retention on unsubstituted Sepharose; it can be shown that this introduces no significant changes in the thermodynamic data to be discussed in the following sections.

Retention on octyl-Sepharose

Influence of the octyl concentration of the gel

Earlier literature reports on the retardation of small model compounds on octyl-agarose seem to disagree. Thus, Hjertén¹⁷ stated that such compounds are not retarded, whereas we found¹⁸ significant retardation of tripeptides such as trileucine. However, the discrepancy might result from the fact that we used octyl-Sepharose CL-4B with a high octyl content (about 200 mmole/mole galactose) whereas Hjertén

used alkyl-agaroses with considerably lower octyl contents (less than 125 mmole/mole galactose). Therefore, we measured the retentions of hexanoic and heptanoic acids on octyl-agaroses with different octyl concentrations. Fig. 1, where $\log K_m$ values are plotted against the degree of octyl substitution, p , clearly indicates that at $p \approx 0.20$ (the degree of substitution of octyl-Sepharose CL-4B) a simple bimolecular association mechanism is not operative and provides an argument for the treatment of our experimental data in terms of a partition model (see Theoretical).

Influence of pH

For a purely hydrophobic interaction of a non-protolytic solute the pH is not expected to exert any effect. Indeed, it can be seen in Fig. 2 that a change in pH from 3.5 to 7.1 has only a minor effect on the interaction of alcohols with octyl groups on the gel. The slight increase in the strength of interaction with increase in pH could be due to the difference in the compositions of the phosphate buffers used, the salting-out effect of HPO_4^{2-} ions being greater than that of H_2PO_4^- ions.

The interaction of proteins with octyl-agarose is often found to weaken at high pH^{6,19}. In the light of the above results, this cannot be due to a weakening of the hydrophobic interaction *per se*. Other effects of pH, *e.g.*, a pH-induced conformation change of the protein, must be evoked to explain such results.

Influence of the nature of the head group

It can be seen in Fig. 2 that for an equal length of the alkyl chain R, the interaction with octyl groups on the gel is stronger for a carboxylic acid RCOOH than for an alcohol ROH.

In a paper on solvophobic interactions, Horváth *et al.*²⁰ derived a theoretical expression for the free energy change on transfer of an amphiphile from solution to the neighbourhood of hydrocarbonaceous ligands of bonded phases. This expression fails to explain the difference we find for the free energy change for alcohols and carboxylic acids of equal chain length. In this instance, the solvophobic theory predicts a difference originating mainly from the electrostatic contribution to the free energy change. This contribution, for an uncharged solute, is governed by its static dipole moment. However, alcohols and carboxylic acids have nearly the same values (1.65 and 1.75 Debye, respectively).

The difference might be explained by assuming that the hydrophilic hydration of an OH group is stronger than that of a COOH group. The hydrophilic hydration sphere of these head groups possibly extends over the nearest CH_2 groups of the alkyl chain, preventing hydrophobic interaction of that part of the chain with octyl groups of the gel, the effect being stronger for an alcohol than for a carboxylic acid.

In Fig. 6 our ΔG° values are plotted against n for the transfer of alcohols (pH 7.10) and carboxylic acids (pH 3.52) from water to octyl-agarose together with literature data (recalculated to convert them to a common molality basis) for the transfer from water to heptane, octane, octanol and the pure liquid. It can be seen that the free energy state of these uncharged solutes in the octyl gel closely resembles that in octanol. In other words, the distribution constants in the system octanol-water are to a great extent indicative of the retention behaviour on octyl-agarose: $K_m \approx k_{\text{octanol-water}}$. (For the retention on alkylsilica from aqueous solution it has been found²¹ that $K_m = aK_{\text{octanol-water}}$, with $a > 1$).

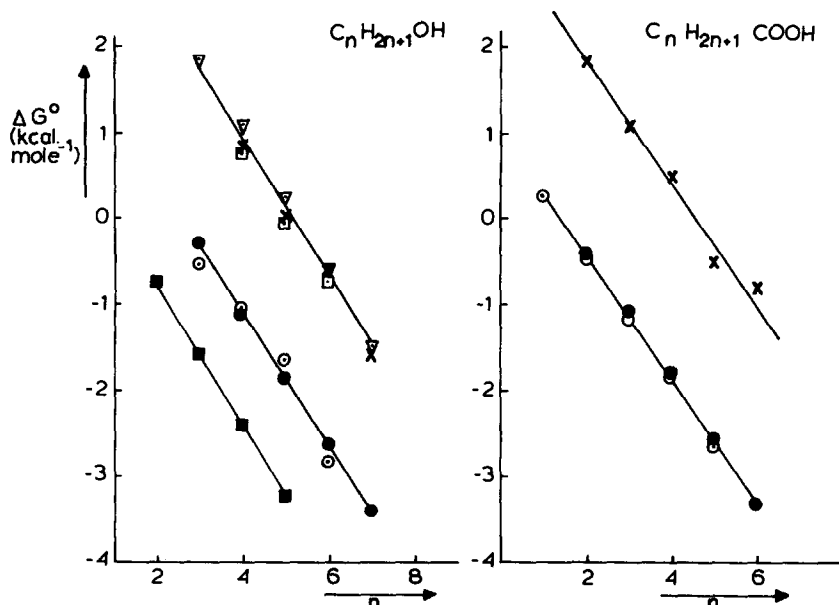


Fig. 6. Standard free energy change for the transfer of alcohols and carboxylic acids from water to octyl agarose (●, this work), from water to hexane (×, ref. 22), from water to octane (□, ref. 23; ▽, ref. 24), from water to pure liquid (■, ref. 25) and from water to octanol (○, ref. 26).

In octanol solution the free energy state of the test compounds is determined by hydrophobic interactions of their alkyl chains with alcohol octyl groups and by hydrogen bonding of the head groups with octanol hydroxyls. The above finding strongly suggests that in chromatography on octyl-Sepharose, in addition to hydrophobic interactions, hydrogen bonding of the head groups also takes place, presumably with hydroxyls of the spacer or of the agarose matrix.

Influence of chain length

As expected for a mainly hydrophobic interaction, lengthening of the alkyl chain increases the extent of interaction with octyl groups on the gel for each series of measurements (Fig. 2). The fact that the lines in Fig. 2 are straight and nearly parallel is plausible. It indicates that, if sufficiently distant from the polar head group (see *Influence of the nature of the head group*), each CH_2 group of the alkyl chain gives a constant contribution to the free energy change, independent of pH and the nature of the head group. This is found in many processes in which hydrophobic interactions play a dominant role²⁷.

The value of the free energy change for multimolecular association per methylene group, $\Delta\Delta G^\circ(\text{CH}_2)$, depends only slightly on the nature of the process studied, as can be seen in the following compilation²⁷ (all values in kcal mole^{-1}):

micelle formation from amphiphiles in water	-0.70
association of amphiphiles with proteins	-0.80
adsorption of amphiphiles to water-paraffin oil interface	-0.81
partition of amphiphiles between organic solvents and water	-0.83
dissolution of hydrocarbons in water	-0.88

TABLE IV

VALUES OF $\Delta\Delta G^\circ(\text{CH}_2)$ FOR RETENTION OF ALCOHOLS AND CARBOXYLIC ACIDS ON ALKYL-SUBSTITUTED AGAROSE AND SILICA

Homologous series	Eluent	pH	Temp. ($^\circ\text{C}$)	Chromatographic support	$\Delta\Delta G^\circ(\text{CH}_2)$ (kcal mole $^{-1}$)	Ref.
Carboxylic acids	25 mM phosphate buffer	3.52	25	Octyl-agarose	0.74 \pm 0.02	This work
Alcohols		3.52			0.76 \pm 0.02	
Alcohols		7.10			0.77 \pm 0.01	
Alcohols	Water	7	27.5	Hexylsilica	0.75	28
Alcohols				Decylsilica	0.81	
Several compounds	100 mM phosphate buffer	2.15	25	Several alkylsilicas	0.82 \pm 0.02	29

In Table IV values of $\Delta\Delta G^\circ(\text{CH}_2)$ resulting from our measurements are compared with those derived from HPLC measurements on alkylsilicas.

The values in Table IV are all very nearly the same, and nearly equal to those given above. However, in view of the barely differing values of $\Delta\Delta G^\circ(\text{CH}_2)$ given above for very different processes, these nearly identical values for retention on alkyl-agarose and alkylsilica cannot be taken as proof of a similar retention mechanism on the two chromatographic supports (there is increasing evidence^{30,31} that the retention on alkylsilica, at least from partly aqueous eluents, should be described in terms of adsorption on an alkyl layer).

Values of $\Delta\Delta G^\circ(\text{CH}_2)$ for processes that can only be conceived in terms of bimolecular association, *e.g.*, dimerization of carboxylic acids in water³² and ion pair formation from alkylammonium and carboxylate ions in water³², are considerably different from the above-mentioned values (about -0.30 kcal mole $^{-1}$). This supports the conclusion we arrived at under *Influence of the octyl concentration of the gel*, namely that a bimolecular association mechanism is not operative in hydrophobic interactions of our test compounds with octyl-Sepharose. It implies that the octyl group density on the agarose fibres is, at least locally, fairly high.

Influence of temperature and thermodynamics of the retention process

General survey. Processes in which hydrophobic interactions play an important role are generally characterized by relatively large and negative values of the standard heat capacity change, ΔC_p° , and by relatively large and positive values of the standard entropy change, ΔS° . These effects have been ascribed to the thermally labile hydrophobic hydration structures and to a general decrease in order on interaction, respectively. It can be seen in Table III that most of the ΔC_p° data are negative and virtually all of the ΔS° data are positive.

As the contribution of hydrophobic interactions to the retention increases with increasing length of the alkyl chain of the solute, one would expect ΔC_p° and ΔS° to become increasingly negative and positive, respectively, with increasing chain length. This is also clearly born out in the results.

In the Introduction, the general rule for hydrophobic interactions, $\Delta H < T\Delta S$,

was mentioned. In Fig. 4, where that standard values ΔH° and $T\Delta S^\circ$ are plotted against chain length, it can be seen that for the three series this rule is obeyed the better, the longer is the alkyl chain. This is to be expected, as the contribution of hydrophobic interactions increases with increasing chain length.

Heat capacity values. The generally negative values, decreasing with increasing chain length, are in good agreement with the order-disturbance concept of hydrophobic interactions. For the alcohol (pH 7.10) and the carboxylic acid series, the mean contributions of a methylene group to the standard heat capacity change, $\Delta\Delta C_p^\circ(\text{CH}_2)$, are about -30 and -25 $\text{cal mole}^{-1} \text{ }^\circ\text{K}^{-1}$, respectively. These values are more negative than the value (-20 $\text{cal mole}^{-1} \text{ }^\circ\text{K}^{-1}$) reported by Konicek and Wadsö³³ for the transfer of a CH_2 group from water to pure alcohol and carboxylic acid. However, as can be seen in Fig. 7, where our ΔC_p° values are compared with literature values pertaining to the transfer of these solutes from water to the pure liquids, the discrepancy stems mainly from results with short alkyl chain lengths, which are less accurate.

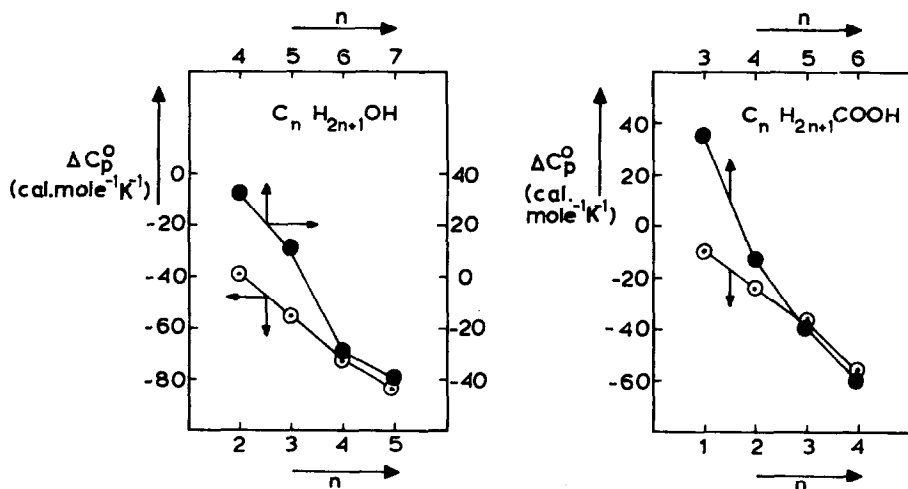


Fig. 7. $\Delta C_p^\circ(\text{CH}_2)$ versus chain length for the transfer of alcohols and carboxylic acids from water to octyl-agarose (●, this work) and from water to the pure liquids (○, refs. 33 and 34).

Enthalpy values. Fig. 4 shows that ΔH° for the alcohols (at pH 3.52 and pH 7.10) is positive, decreasing with increasing chain length. For the carboxylic acids ΔH° also decreases with increasing chain length from slightly positive to slightly negative values. The contributions of a CH_2 group to the standard enthalpy change, $\Delta\Delta H^\circ(\text{CH}_2)$, are almost identical in the three series for $n \geq 4$, being about -0.3 kcal mole^{-1} . This value is in fair agreement with that pertaining to the transfer of these solutes from water to the pure liquids, as is demonstrated in Fig. 8, where our ΔH° values are compared with literature data, recalculated to convert them to a common molality basis.

About the same contribution of a methylene group was found^{23,35} in the enthalpy of transfer of these solutes from water to *n*-octane (-0.39 and -0.28 kcal mole^{-1} for alcohols and carboxylic acids, respectively) and in the enthalpy of transfer

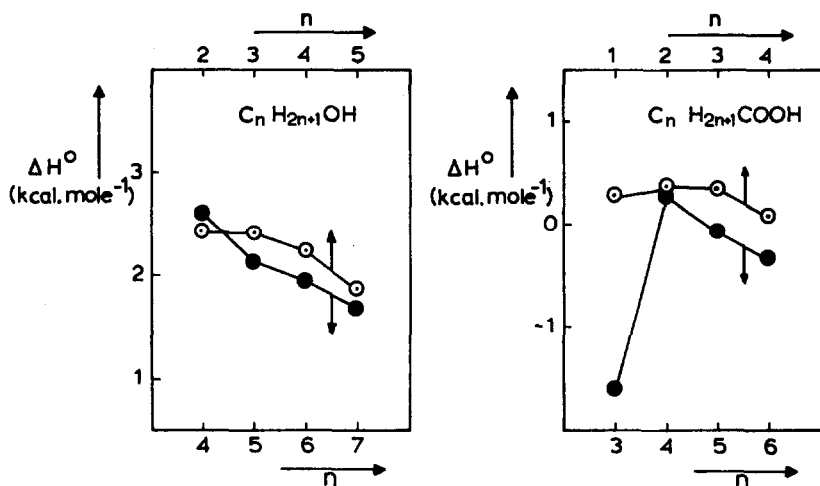


Fig. 8. $\Delta H^\circ(CH_2)$ versus chain length for the transfer of alcohols and carboxylic acids from water to octyl-agarose (●, this work) and from water to the pure liquids (○, refs. 33 and 34).

of homologous aliphatic ketones and esters from water to cyclohexane³⁶ (-0.26 kcal mole $^{-1}$).

Entropy values. Fig. 4 shows that ΔS° values are positive for the three series and increase with increasing chain length. The contribution of a methylene group to the standard entropy change, $\Delta \Delta S^\circ(CH_2)$, is almost the same in the three series for $n \geq 4$, being about 1.5 and 1.9 cal mole $^{-1} \text{ }^\circ K^{-1}$ for the alcohols at pH 3.52 and pH 7.10, respectively, and about 1.4 cal mole $^{-1} \text{ }^\circ K^{-1}$ for the carboxylic acids. Again, these values compare favourably with literature values pertaining to the transfer of alcohols from water to the pure liquids and of carboxylic acids from water to *n*-octane, as is demonstrated in Fig. 9.

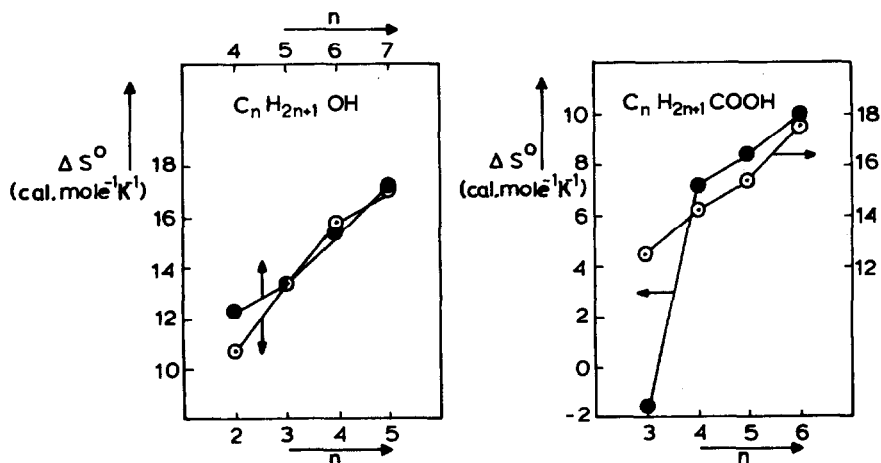


Fig. 9. $\Delta S^\circ(CH_2)$ versus chain length for the transfer of alcohols and carboxylic acids from water to octyl-agarose (●, this work), of alcohols from water to the pure liquids (○, ref. 25) and of carboxylic acids to *n*-octane (○, ref. 35).

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