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MECHANISM OF SEPARATION OF ALIPHATIC ALCOHOLS IN AQUEOUS DEXTRAN GEL SYSTEMS

KIKUJIRO UJIMOTO* and HIRONDO KURIHARA
Department of Chemistry, Faculty of Science, Fukuoka University, Nanakuma 11, Nishi-ku, Fukuoka 814-01 (Japan)
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SUMMARY

The gel chromatographic behaviour of aliphatic alcohols on Sephadex G-10 and G-15 in aqueous systems was elucidated primarily in terms of hydrophobic interactions by use of the thermodynamic functions for dissolution in water and for partition in gel chromatography; the former was obtained from the literature and the latter from the temperature dependence of the distribution coefficient (K_d).

From an enthalpy–entropy compensation test for partitioning processes of solutes between the mobile and stationary phases of gel columns, it was found that methanol, ethanol, 2-propanol and 2-methyl-2-propanol behaved in a different manner to the primary *n*-alkanols containing more than two carbon atoms in the molecule did. The salting-out effect of the alcohols from the bulk to the gel phase and the effect of addition of methanol to water as the eluent are discussed.

INTRODUCTION

Although solutes are separated in gel chromatography principally depending on their size, the behaviour of relatively small molecules and ions is often affected anomalously by several kinds of side-effects such as electrostatic and hydrophobic interactions and hydrogen bonding on tightly cross-linked gels in aqueous systems. The separation mechanism with such side-effects has been studied by investigating the dependence of elution volumes or volumetric distribution coefficients (*e.g.*, K_d or K_{av}) on sample concentration, type of eluent, eluent concentration, pH of eluent, temperature, etc.[1].

Hydrophobic interactions, one of the important side-effects, have been widely observed for organic, biochemical and biological solutes and have sometimes been utilized to fractionate proteins[1]. However, they have been investigated mostly by phenomenological methods. For example, Morris[2] contended that chromatographic behaviour can be ascribed mainly to a hydrophobic interaction only if all of the following three criteria are met: (1) hydrophobic sites can be identified on the stationary phase; (2) the solute is more firmly adsorbed at higher than lower temperatures, so that the free energy of adsorption is primarily entropic in nature; and (3)

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adsorption occurs at relatively high salt concentrations and desorption at lower salt concentrations. An additional criterion[1] has also proposed that adsorption can be depressed by the addition of weakly polar solvents to the aqueous eluents used.

The endothermic temperature dependence of the K_d value or capacity factor is essential to hydrophobic interactions, because these interactions are solvent dependent[3]. The careful examination of the temperature dependence of the K_d values gives thermodynamic functions such as ΔG°_{GC}, ΔH°_{GC} and ΔS°_{GC}, which are the standard free energy, enthalpy and entropy changes, respectively, required for transfer of the solutes from the mobile phase (bulky phase) to the stationary phase (gel phase) at infinite dilution.

Only a few workers[4–11] have dealt with hydrophobic interactions in gel chromatography by the use of the thermodynamic approach, although the effect of temperature on elution volumes and several kinds of volumetric distribution coefficients has been examined as the major criteria for hydrophobic interactions[1,12,13]. Marsden[4,7] studied the affinity of dextran gels for weakly polar solutes in aqueous systems and concluded that water plays the dominant role in producing the entropic affinity in hydrophobic interactions. Janado and co-workers[9–11] reported recently that the preferential partition of water-insoluble dyes and sodium dodecyl sulphate on Sephadex and Bio-Gels can be ascribed primarily to the hydrophobic free energy arising from the anomalous nature of hydrated water in the gel beads. In both instances the ΔG°_{GC} values are negative and the ΔH°_{GC} and ΔS°_{GC} values are positive, indicating that hydrophobic interactions are the main effect. No study, however, has been carried out to clarify the hydrophobic interaction mechanism by correlating the thermodynamic functions for gel chromatographic processes with parameters characteristic of the interaction, *e.g.*, those for hydration of so-called "hydrophobic solutes".

This study, therefore, was undertaken in order to clarify the mechanism of the separations of small hydrophobic solutes on tightly cross-linked dextran gels (Sephadex G-10 and G-15), principally by correlating the ΔS°_{GC} values with the ΔS°_{HY} values, the standard entropy changes for hydration of the solutes. Nine aliphatic alcohols were employed as a homologous series of hydrophobic solutes for two reasons: first, a set of accurate thermodynamic data on the dissolution of the gaseous alcohols into water is available[14–16] and the hydrophobic hydration of these alcohols described in detail; and secondly, the K_d value of methanol is independent of the pH of the eluent on Sephadex G-10, although those of inorganic ions varied significantly with pH[17]. This characteristic of alcohols as non-electrolytes will be advantageous in investigations of the separation mechanism without electrostatic interactions. According to the criteria mentioned above, the effects of salting-out and addition of methanol to water as the eluent were also examined in order to establish whether the gel chromatographic behaviour of the alcohols is primarily governed by the hydrophobic interactions.

For the purpose of the differentiation of solutes in mechanistically different processes, an enthalpy–entropy compensation test based on a linear free-energy relationship[18–20] was applied to the partitioning of solutes in gel chromatography.

EXPERIMENTAL

Sample solutions

All reagents used were of guaranteed reagent grade from Wako (Osaka, Japan) or Nakarai Chemicals (Kyoto, Japan), unless otherwise stated. Methanol, ethanol, 1-

propanol and 1-butanol labelled with carbon-14 were obtained from New England Nuclear (Boston, MA, U.S.A.) and used as radiotracers.

Sample solutions for aqueous systems were prepared by dissolving methanol (MeOH), ethanol (EtOH), 1-propanol (1-PrOH), 1-butanol (1-BuOH), 1-pentanol (1-PeOH), 1-hexanol (1-HexOH), 1-heptanol (1-HepOH), 2-propanol (*i*-PrOH) and 2-methyl-2-propanol (*t*-BuOH) in the eluents. The sample concentration was $5 \cdot 10^{-2} M$, except for 1-HexOH and 1-HepOH, of which saturated solutions were used because of their poor solubilities. For MeOH, EtOH, 1-PrOH and 1-BuOH, sample solutions of concentration 0.01, 0.1, 0.2 and 0.4 M were also employed in order to examine the effect of sample concentration on the K_c values.

Blue dextran 2000 (Pharmacia, Uppsala, Sweden; 0.36%) and tritiated water (New England Nuclear) were used as standard materials with $K_d = 0$ and 1, respectively. Sodium-22 or chloride-36 ion was employed as an internal standard for both of 1-PrOH and 1-BuOH with 0.1 M sodium chloride eluent at pH 2 or 12.

In experiments with the mixed eluents containing water and methanol, sample solutions of 1-octanol (1-OcOH) in the eluents at a concentration of $5 \cdot 10^{-2}$ M were also used in addition to those of the alcohols stated above. In this instance, polyethylene oxide (Toyo Soda Manufacturing, Tokyo, Japan; $\bar{M}_w = 1.5 \cdot 10^5$, $\bar{M}_w/\bar{M}_n = 1.04$) was employed instead of Blue Dextran 2000.

Eluents

The aqueous eluents used were distilled water and 0.1 M sodium chloride solution at pH 2 or 12 adjusted with hydrochloric acid or sodium hydroxide solution. Sodium chloride solutions of concentration 0.01, 0.05, 0.2, 0.5 and 1.0 M at pH 2 were also employed to examine the effect of eluent concentration on the K_d values of the alcohols.

The mixed water–methanol eluents contained 50, 90 and 95% of methanol.

Columns and elution procedure

Sephadex G-10 or G-15 (Pharmacia, dry particle size 40–120 μm) was packed into the column (Pharmacia, K16/100) with flow adaptors at both the top and bottom, as described in a previous paper[21].

All the equipment except the recorder (R-201, Rikadenki Kogyo, Tokyo, Japan) and cooling bath (PBC-4,Neslab Instruments, NH, U.S.A.) were installed in an M-1900E chromatochamber (Toyo Kagaku Sangyo, Osaka, Japan), which was thermostated with an accuracy of ± 1°C. On investigating the temperature dependence of the K_d values, the column and the reservoir were water-jacketed and the elutions were carried out at 10, 15, 20, 25 and 30 $\pm$ 0.02°C by the use of a TC-3 thermostat (PM. Thamson, B.V., Zoetermeer, The Netherlands) combined with the cooling bath.

A 1-cm^3 volume of the sample solution was introduced on to the column with a hypodermic syringe through a line sample injector of the septum type. The elution was allowed to proceed at a constant flow-rate of 60 cm^3/h with a Duramat pump (Chemie u. Filter, Heidelberg, G.F.R.) or a pump of the single-plunger type (KHU-16, Kyowa Seimitsu, Tokyo, Japan) combined with a KU-1 damper (Kyowa Seimitsu). The effluent from the column was collected in 1-cm^3 fractions with an Ultrorac 7000 fraction collector (LKB, Bromma, Sweden).

The effluent was also monitored continuously with a differential refractometer (Showa Denko, Tokyo, Japan, Shodex RI, SE-11), which was connected to the recorder. The activities of tritiated water and ^{14}C-labelled alcohols were measured with a liquid scintillation spectrometer (Packard Instrument, IL, U.S.A., Model 3320 or 2660), and those of $^{22}Na^+$ and $^{36}Cl^-$ with an auto-gamma scintillation spectrometer (Packard, Model 5230) or an automatic GM counter (LBC-22B, Aloka, Mitaka, Japan). The dead volume from the cell of the refractometer to the fraction collector was determined by comparing the elution volumes of the radioactive alcohols obtained by activity measurements with those obtained by refractometry, and the elution volumes of the alcohols were corrected by this value when calculating the K_d values.

Calculation of the K_d value

The K_d value was obtained by the equation[21]

$$K_d = (V_e - V_0)/(V_t - V_0) \tag{1}$$

or

$$K_d = K_{d(IS)}\,(V_e - V_0)/(V_{IS} - V_0) \tag{2}$$

where V_t is the total liquid volume of the gel column, V_0 the void volume outside the gel, V_e and V_{IS} the elution volumes of the sample and the internal standard, respectively, and $K_{d(IS)}$ the distribution coefficient of the internal standard. The elution volume of tritiated water was used as the V_t value, that of Blue Dextran 2000 or polyethylene oxide as V_0 and that of $^{22}Na^+$ or $^{36}Cl^-$ as V_{IS} for the elutions of 1-PrOH and 1-BuOH at pH 2 or 12, respectively.

Calculation of the thermodynamic functions

The K_d value obtained dynamically by the column method has been accepted as a parameter representing thermodynamic equilibrium in gel chromatography, because this is usually equal to the static value obtained by a batch method[22]. Considering that the dynamic K_d value corresponds to the equilibrium constant based on partitioning of the solute between the gel phase and the bulky phase outside the gel particles, the thermodynamic functions can be obtained by the equation

$$\ln K_d = (-\Delta H^\circ_{GC}/RT) + (\Delta S^\circ_{GC}/R) \tag{3}$$

where ΔH°_{GC} denotes the standard enthalpy change, ΔS°_{GC} the standard entropy change required to transfer 1 mol of the solute from the bulk to the gel phase, R the gas constant and T absolute temperature. When the plot of $\ln K_d$ against $1/T$ is linear, ΔH°_{GC} can be obtained from the slope and ΔS°_{GC} from the intercept.

RESULTS AND DISCUSSION

Effect of sample concentration

The dependence of the K_d values on sample concentration suggests that two factors are operative in gel chromatographic processes: (1) the distribution isotherms

are non-linear and dependent on concentration, and (2) the solutes undergo molecular changes such as association, dissociation, complex and ion-pair formation and adsorptive properties depending on ionic strength[1]. Therefore, a study of the effect of sample concentration may provide information about such side-effects.

The effect of the sample concentration was examined over the range 0.01–0.4 *M* for MeOH, EtOH, 1-PrOH and 1-BuOH by the use of radioactive labelling on Sephadex G-10 with 0.1 *M* sodium chloride solution as eluent at pH 2. The results are given in Table I, and indicate that the K_d values of the individual alcohols were not affected by the variation in the sample concentration over the range studied. This tendency is also consistent with the observation by Marsden[4] that the behaviour of non-electrolytes was independent of the concentration over wide ranges in aqueous dextran gel systems.

TABLE I
EFFECT OF SAMPLE CONCENTRATION ON K_d VALUES
Gel, Sephadex G-10; eluent, 0.1 *M* NaCl at pH 2, temperature, 20.5 ± 0.3°C.

Sample	*Concentration (M)*				
	0.01	0.05	0.1	0.2	0.4
MeOH	0.76_1	0.75_8	0.76_6	0.76_3	0.76_0
EtOH	0.76_1	0.76_2	0.76_4	0.76_2	0.76_9
1-PrOH	0.89_5	0.89_3	0.88_9	0.90_2	0.89_6
1-BuOH	1.10_6	1.09_8	1.10_7	1.10_2	1.10_5

In conclusion, the two factors mentioned above had a negligible effect in gel chromatographic processes in this study. In further experiments, sample solutions at 0.05 *M* were used except for 1-HexOH and 1-HepOH with the aqueous eluents, for which saturated solutions were employed.

Effect of eluent concentration

It is well known that the elution volumes of organic non-electrolytes such as oligosaccharides, proteins and other biological substances vary considerably depending on the concentrations of inorganic and organic electrolytes in the eluent[1]. These phenomena were often discussed qualitatively in terms of salting-out or salting-in effects. When a salting-out phenomenon was observed, it was accepted as a criterion that the hydrophobic interaction between the solute and the gel matrix may be operative as one of side-effects in gel chromatography[1].

Therefore, the dependence of the K_d values on the eluent concentration was examined for MeOH, EtOH, 1-PrOH and 1-BuOH on Sephadex G-10 by using sodium chloride solutions of concentration of 0–1.0 *M* at pH 2 as the eluents. The results are shown in Fig. 1.

The K_d values of the individual alcohols are almost constant below an ionic strength of 0.1 *M* and tend to increase gradually with increase in ionic strength, especially above 0.2 *M*. This effect is more significant for 1-PrOH and 1-BuOH than for MeOH and EtOH, the K_d values of which are only slightly affected. Marsden[7] observed that guanidinium sulphate, potassium chloride and magnesium chloride

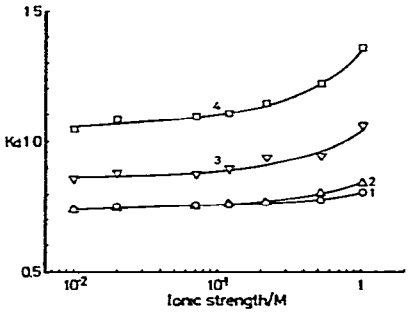

Fig 1. Effect of eluent concentration Gel: Sephadex G-10. Eluent. NaCl solutions of various concentration at pH 2. Temperature· 20.8 ± 0 5°C. 1 = MeOH, 2 = EtOH; 3 = 1-PrOH; 4 = 1-BuOH.

increase the K_{av} value of 1-PeOH. Hence, the obvious tendency for the salting-out effect to occur with 1-PrOH and 1-BuOH in this study suggests that the hydrophobic interaction operates possibly as one of the important factors governing the behaviour of the primary *n*-alkanols containing more than two carbon atoms in the molecule in gel chromatographic processes. The mechanism of the salting-out phenomenon will be discussed in terms of the K_d values of the eluent ions (See *Separation mechanism*).

Effect of addition of methanol to water as eluent

Organic solutes in uncharged states tend generally to be adsorbed anomalously with aqueous eluents on the gels of the Sephadex G and LH types, and to be desorbed with non-polar and weakly polar eluents[1]. With the latter eluents, the elution volume is linearly dependent on the logarithm of the molecular weight or of the molar volume of the solute. This phenomenon has been interpreted mostly in terms of the molecular sieving effect, depending on the size of the solute molecule.

The effect of the addition of methanol to an aqueous eluent on the K_d values was examined for ten aliphatic alcohols on Sephadex G-10 at 20°C. The K_d values are shown as a function of logarithm of molecular weight in Fig. 2. The K_d value decreases with increasing amount of methanol added in each instance and then the K_d value for each alcohol with 90% methanol becomes identical with that with 95% methanol, with which the linear dependence of the K_d values on the logarithm of molecular weight was observed, suggesting that molecular sieving is predominantly operative in the separation mechanism studied. However, the linear relationship for the group of MeOH, EtOH, *i*-PrOH and *t*-BuOH (classified into group B) is different from that for the other primary *n*-alkanols (group A). This might be attributable to the different extents of the interaction between the solutes and water and/or methanol contained in the eluent.

The K_d values of members of group A with pure water increase markedly with increasing molecular weight, whereas the K_d values of group B are slightly reduced, suggesting that side-effects possibly operate as dominant factors for group A. One of them may be hydrophobic interactions. With 50% methanol, the behaviour of the alcohols may be governed by both the hydrophobic interactions and the molecular sieving effect to various extents in each instance.

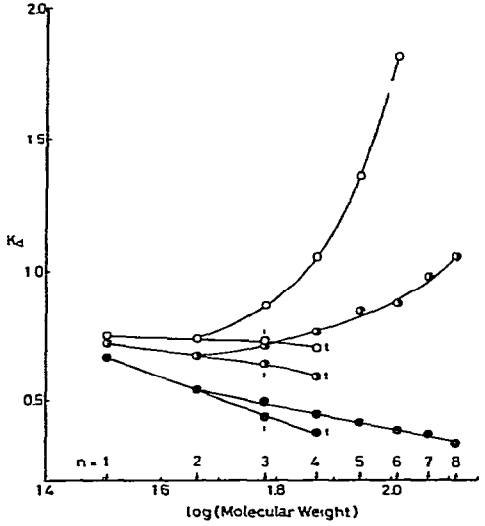

Fig 2 Effect of addition of methanol to water as eluent. Gel Sephadex G-10 Temperature: 20°C Eluent ○, water; ◑, 50% methanol; ●, 90 and 95% methanol. n is the number of carbon atoms in the alcohol molecule; i and t denote iso and tertiary, respectively

Effect of temperature

When the hydrophobic interaction is primarily operative, volumetric parameters such as elution volumes and K_d values generally increase with increase in temperature[1]. Without side-effects, the volumetric parameters are insensitive to temperature variation[1]. Therefore, the temperature dependence of the K_d values was examined for the aliphatic alcohols on Sephadex G-10 and G-15 over the range 10–30 or 10–35°C. The ln K_d values under different conditions are shown as a function of the reciprocal of absolute temperature in Fig. 3.

With the aqueous eluents, the K_d values for all of the alcohols increase with increasing temperature (endothermic, Fig. 3a–d), whereas those with 95% methanol are independent of temperature, within experimental error (Fig. 3e). These observations suggest the possibility that the hydrophobic interaction may be an important factor for alcohols in the aqueous dextran gel systems. With 95% methanol, the molecular sieving effect may operate predominantly as described in the preceding section.

Evaluation of thermodynamic functions for gel chromatographic process

When tritiated water is used as a standard material for the determination of the total liquid volume of the column, the K_d value is underestimated owing to an isotopic exchange reaction of the hydrogen atoms between the tritiated water and the hydroxyl and/or the carboxyl groups of the gel matrix. Marsden[7,23] reported that the correction factors to the $V_t - V_0$ values due to the isotopic exchange were 1.091 and 1.075 for Sephadex G-10 and G-15, respectively. Even if these factors are introduced into the calculation of the thermodynamic functions, the ΔH°_{GC} values are invariable and the ΔS°_{GC} should be reduced only by 0.724 and 0.601 $J°K^{-1}$ mol^{-1} on Sephadex G-10 and G-15 in each instance, provided that the factors are independent of tem-

perature over the range studied. As this assumption was not examined, the contribution of the correction factors to the ΔS°_{GC} values was not taken into account in the present study.

On the other hand, when the thermodynamic functions are evaluated on the basis of Van 't Hoff plots, it is essential that the properties of the gel do not undergo any change over the temperature range investigated. Lampert and Determann[24] observed the temperature dependence of swelling of soft gels, such as Sephadex G and LH and Bio-Gel P types. According to preliminary experiments[25], Sephadex G-10

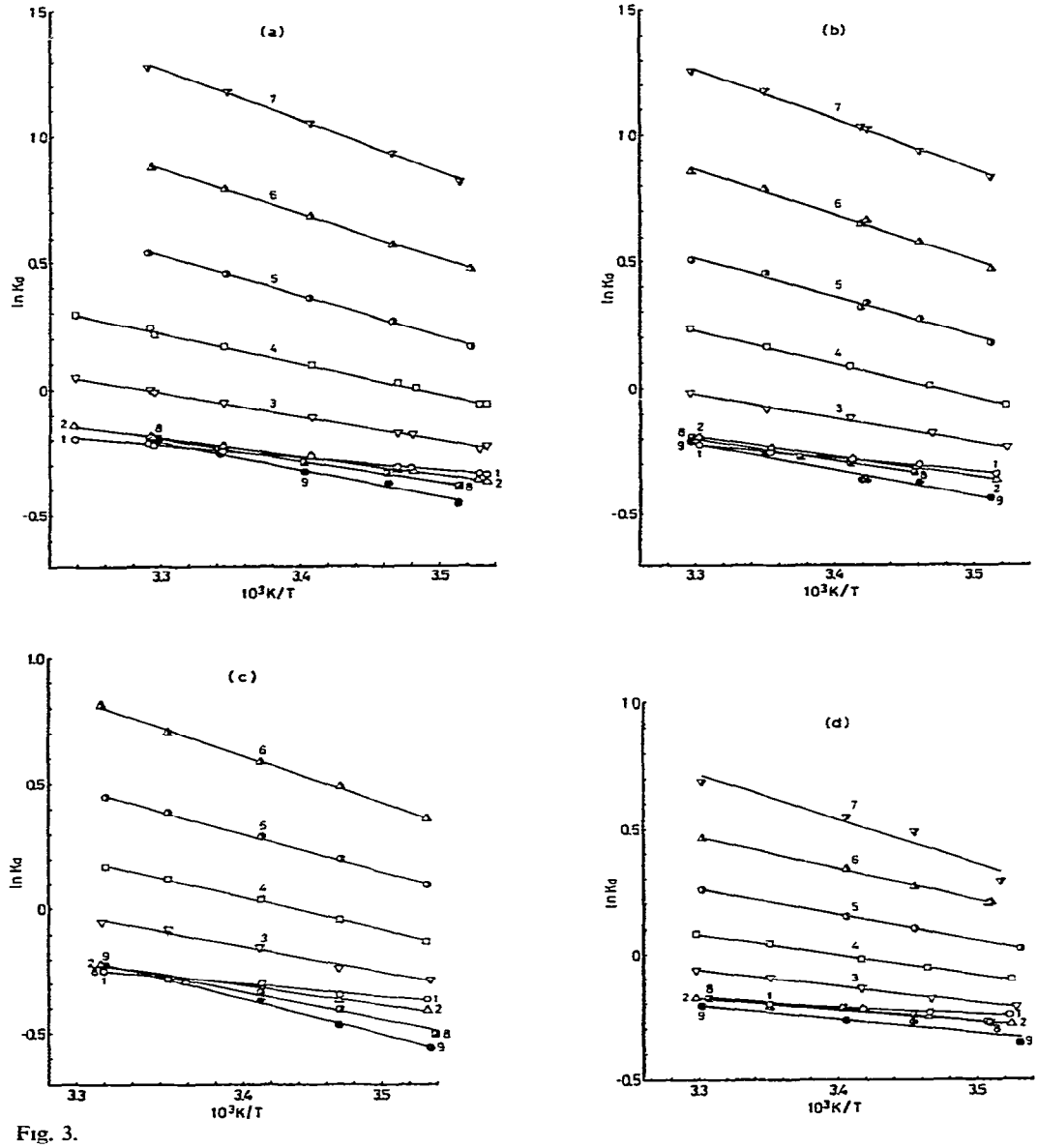

Fig. 3.

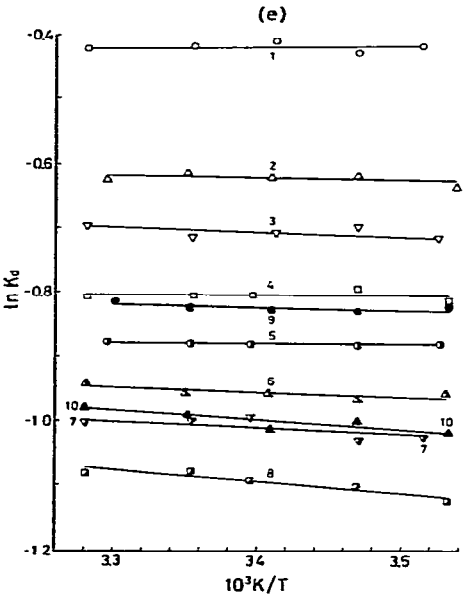

Fig. 3. Van 't Hoff plots of ln K_d against $1/T$ (a) on Sephadex G-10 with 0.1 *M* NaCl at pH 2, (b) on Sephadex G-10 with 0.1 *M* NaCl at pH 12; (c) on Sephadex G-10 with water, (d) on Sephadex G-15 with 0.1 *M* NaCl at pH 2; (e) on Sephadex G-10 with 95% methanol. In (a)–(d): 1 = MeOH; 2 = EtOH; 3 = 1-PrOH; 4 = 1-BuOH; 5 = 1-PeOH; 6 = 1-HexOH; 7 = 1-HepOH; 8 = *t*-PrOH; 9 = *t*-BuOH In (e) 8 = 1-OcOH; 9 = *t*-PrOH; 10 = *t*-BuOH

columns swollen in water and in 0.1 *M* sodium chloride solution at 10°C shrunk by 2.7 and 5.6%, respectively, of their bed volumes when the temperature increased to 30°C, and the coefficients to transform the K_d into the K_{av} (F_g) decreased only 3.2 and 3.7%, respectively. With 95% methanol, the bed volume of the column was almost independent of temperature and the F_g value decreased only 6.5% when the temperature increased from 10 to 30°C.

Moreover, as the Van 't Hoff plots shown in Fig. 3 yielded a good linear relationship over the temperature range examined in each instance, the effect of temperature on the gel properties was also neglected.

Enthalpy–entropy compensation test for gel chromatographic process

According to an extrathermodynamic approach* based on a linear free-energy relationship, enthalpy–entropy compensation implies a linear dependence of the overall free-energy changes on the corresponding enthalpy changes for intrinsically similar physico-chemical processes. If a physico-chemical change fails to conform to the common compensation pattern, it is assumed to be different from the common process in some mechanistic details. The enthalpy–entropy compensation test, therefore, can serve as a diagnostic tool for differentiating among the solute behaviour in mechanistically different processes[20].

* Without a rigorous thermodynamic foundation.

However, the linearity of the plot of ΔH°_{GC} against ΔS°_{GC} for the processes to be elucidated does not always indicate the existence of compensation behaviour based on the common physico-chemical process, because compensation may arise in part from statistical effects due to errors associated with the determination of ΔH°_{GC} and ΔS°_{GC} values[18,19]. In order to minimize the statistical effects, use of the following relationship was recommended for elucidating compensation behaviour, assuming that the K_d value corresponds to the thermodynamic equilibrium constant in gel chromatographic partitioning of the solutes[20]:

$$\ln K_{d(T)} = -\frac{\Delta H^{\circ}_{GC}}{R}\left(\frac{1}{T}-\frac{1}{\beta}\right)-\frac{\Delta G^{\circ}_{\beta}}{R\beta} \tag{4}$$

where $K_{d(T)}$ denotes the distribution coefficient at the reference temperature, T, and ΔG°_{β} the free-energy change of a physico-chemical interaction at the compensation temperature, β. When $T = \beta$, a homologous series of solutes in a common process has

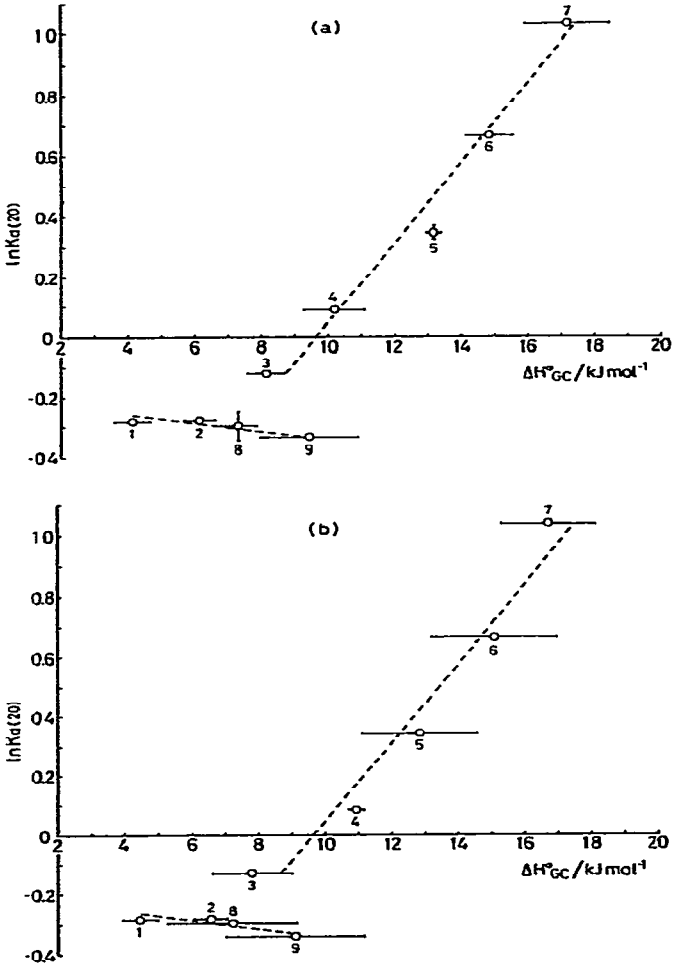

Fig. 4.

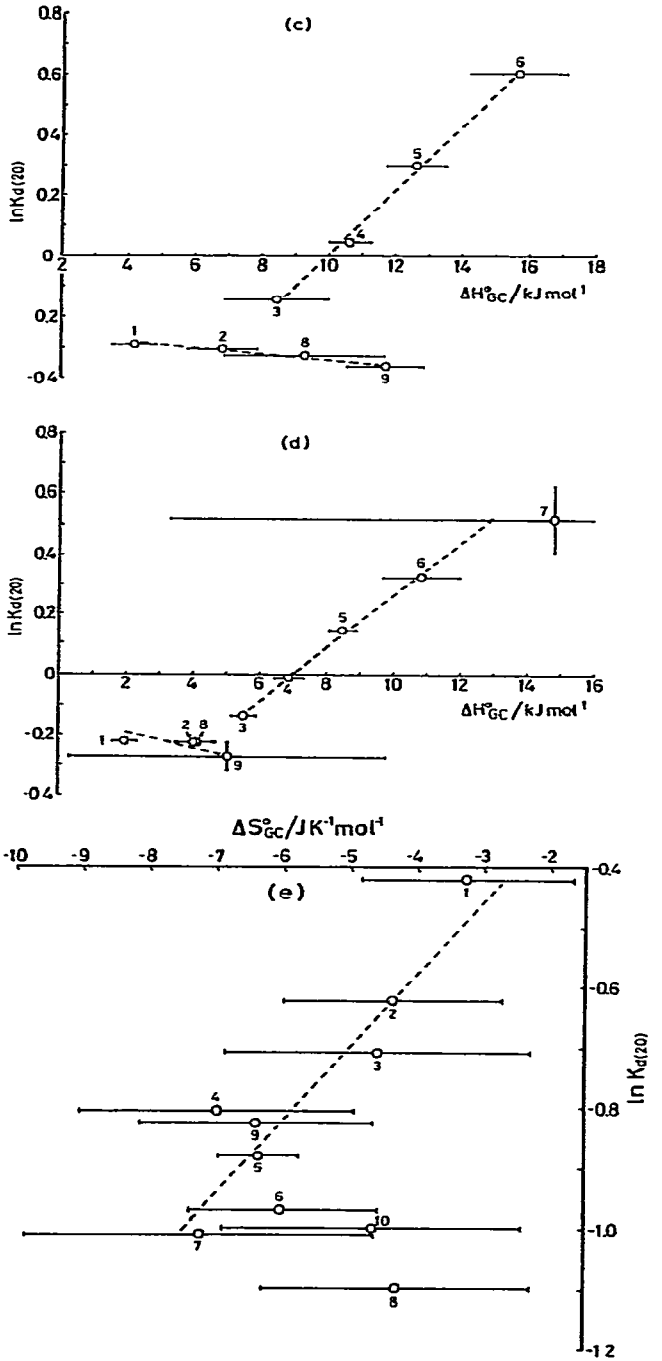

Fig. 4. Plots for enthalpy–entropy compensation test. Key as in Fig. 2. A solid line across a circle indicates the 0.95 confidence interval. In (e), it is the 0.80 confidence interval, because of a relatively large experimental error. Broken lines demonstrate the linear regression lines for groups A and B in each instance.

the same value of ΔG°_{β}, because the changes in ΔH°_{GC} are offset by those in $T\Delta S^{\circ}_{GC}$. As the reference temperature, T, the temperature near the harmonic mean of the experimental temperatures for the evaluation of the ΔH°_{GC} values is usually employed in order to reduce the statistical errors[20]. Hence, 20°C was tentatively employed under the present experimental conditions. Plots of ln $K_{d(20)}$ against ΔH°_{GC} are shown in Fig. 4, where the ln $K_{d(20)}$ values were plotted against ΔS°_{GC} with 95% methanol (Fig. 4e), because the confidence intervals of ΔS°_{GC} were less than those of ΔH°_{GC} in this instance.

First, it should be noted that with the aqueous eluents the compensation behaviour of members of group A is markedly different from that of group B, regardless of both the type of eluent and the gel porosity. This indicates that there may be a significant difference in the separation mechanisms for the aliphatic alcohols between groups A and B, which will be discussed in terms of different modes of hydration in the following section.

Secondly, the compensation pattern with 95% methanol obviously differs from those with the aqueous eluents in two important respects: all of the ΔS°_{GC} values with the former eluent are negative whereas with the latter they are positive, and no appreciable difference of the compensation behaviour between groups A and B was observed with the former, although plots for 1-OcOH and *t*-BuOH deviate considerably owing to experimental error. The characteristic behaviour with the eluent almost free from water is attributable principally to the molecular sieving effect, as discussed above.

Table II summarizes the results obtained by the compensation test for the gel chromatographic behaviour of the alcohols with the aqueous eluents. The β and ΔG°_{β} values were obtained from the slopes and intercepts according to eqn. 4. For group A, the slopes and β values are indistinguishable from one another at the 0.05 confidence level, but the intercept on Sephadex G-15 is different from those on G-10. This suggests that cross-links of the ether type may participate considerably in the separation mechanism. The β values for group A agree well with that reported by Marsden for EtOH, 1-BuOH, 1-PeOH and 1-HexOH on Sephadex G-10 (229°K), suggesting

TABLE II

PARAMETERS OBTAINED BY ENTHALPY–ENTROPY COMPENSATION TEST

Variances of ln K_d and ΔH°_{GC} were not taken into account for the calculation of parameters Values of slopes and intercepts are given with 0 95 confidence intervals

*Group**	*Chromatographic condition***	*Correlation coefficient*	*Slope* 10^3	*Intercept*	β (°K)	ΔG°_{β} (kJ mol^{-1})
A	1	0.988	$0.12_6 \pm 0.03_6$	$-1.1_9 \pm 0\,4$	224	2.2_2
	2	0 976	$0\,13_0 \pm 0.05_3$	$-1\,2_4 \pm 0\,7$	223	$2\,3_0$
	3	0.998	$0\,10_6 \pm 0.02_0$	$-1\,0_6 \pm 0.2$	223	$2\,0_4$
	4***	0 999	$0\,08_7 \pm 0\,01_3$	$-0\,6_1 \pm 0\,1$	242	1.2_3
B	1	−0.884	$-0\,01_0 \pm 0\,01_6$	$-0\,2_3 \pm 0\,1$	301	0.5_7
	2	−0 852	$-0\,01_2 \pm 0\,02_2$	$-0\,2_2 \pm 0\,1$	302	0.5_5
	3	−0.975	$-0.00_9 \pm 0.00_6$	$-0\,2_5 \pm 0.5$	300	$0\,6_2$
	4	−0 717	$-0.01_3 \pm 0\,03_9$	$-0.1_9 \pm 0\,1$	303	0.4_7

* A· 1-PrOH, 1-BuOH, 1-PeOH, 1-HexOH and 1-HepOH. B· MeOH, EtOH, *i*-PrOH and *t*-BuOH

** 1, On Sephadex G-10 with 0.1 *M* NaCl at pH 2; 2, on Sephadex G-10 with 0.1 *M* NaCl at pH 12; 3, on Sephadex G-10 with water; 4, on Sephadex G-15 with 0.1 *M* NaCl at pH 2.

*** The value for 1-HepOH was excluded because of its large experimental error

the important role of water in the gel chromatographic processes for the members of group A[7]. On the other hand, neither the slopes nor the intercepts for group B were statistically distinguishable from one another, but it is not yet clear whether this may be ascribed to the intrinsically similar process or to experimental error.

Separation mechanism

The dependence of the K_d values on temperature and on the concentrations of sodium chloride and methanol in the eluent discussed above suggests strongly that side-effects play a dominant role in the mechanism of separation of the aliphatic alcohols and that the most important factor is possibly hydrophobic interactions.

Marsden[4,7] investigated the behaviour of weakly polar solutes, including eight aliphatic alcohols, on tightly cross-linked dextran gels in aqueous systems and proposed that the hydrophobic interaction between the solutes and the gel matrix may be the most important factor governing the solute behaviour. However, the behaviour of *i*-PrOH and *t*-BuOH was anomalous and could not be interpreted.

Janado and co-workers[9–11] reported recently the preferential partition of water-insoluble dyes and sodium dodecyl sulphate in the gel phases of Sephadex G and of Bio-Gel P-2 in aqueous systems. By investigating this preferential partitioning in terms of the transfer free-energy of the solute from the mobile phase to the swollen gel beads, they claimed that the overall transfer process is primarily governed by hydrophobic free energy arising from the anomalous nature of hydrated water in the gel matrix, *i.e.*, in highly hydrated water so-called "iceberg formation" around the solute molecules is partially prevented and the hydrophobic free energy is accordingly decreased resulting in preferential partition

On the other hand, the hydrophobic hydration of non-polar and weakly polar substances such as noble gases, hydrocarbons and aliphatic alcohols in aqueous solutions has been extensively studied (*e.g.*, ref. 26). Studies on viscosity and maximal density of aqueous solutions of alcohols revealed that alcohol molecules produce hydration shells around themselves, where water is organized more highly than in bulk, *i.e.*, ordering of water structure tends towards an ice-like arrangement. Thermodynamic studies gave the standard free-energy, enthalpy and entropy changes for the dissolution of gaseous alcohols in water; the free-energy changes are positive and the enthalpy and entropy changes are negative. This is consistent with the model proposed in the former studies, as far as the hydrophobic hydration phenomenon is concerned.

In order to discuss the meaning of hydrophobic interactions in gel chromatography, ΔS°_{GC} values were plotted against ΔS°_{HY}[14–16], as shown in Fig. 5. ΔS°_{HY} denotes the standard entropy change on dissolving 1 mol of gaseous alcohol in water at infinite dilution. In each instance, good linear relationships were observed for groups A and B. This phenomenon can be interpreted physico-chemically as follows.

The entropy of the system, including solutes and solvent, decreases on dissolution of gaseous alcohols in water owing to the formation of the hydration shells around the solute molecules, where organization of the structure of water tends towards an ice-like arrangement. The solute in the mobile phase is in such a state, as mentioned. The gel skelton may also organize water around its hydrophobic sites on swelling in water, with an accompanying decrease in entropy due to ordering of the solvent structure. Then, the system will partially regain its entropy loss as a result of

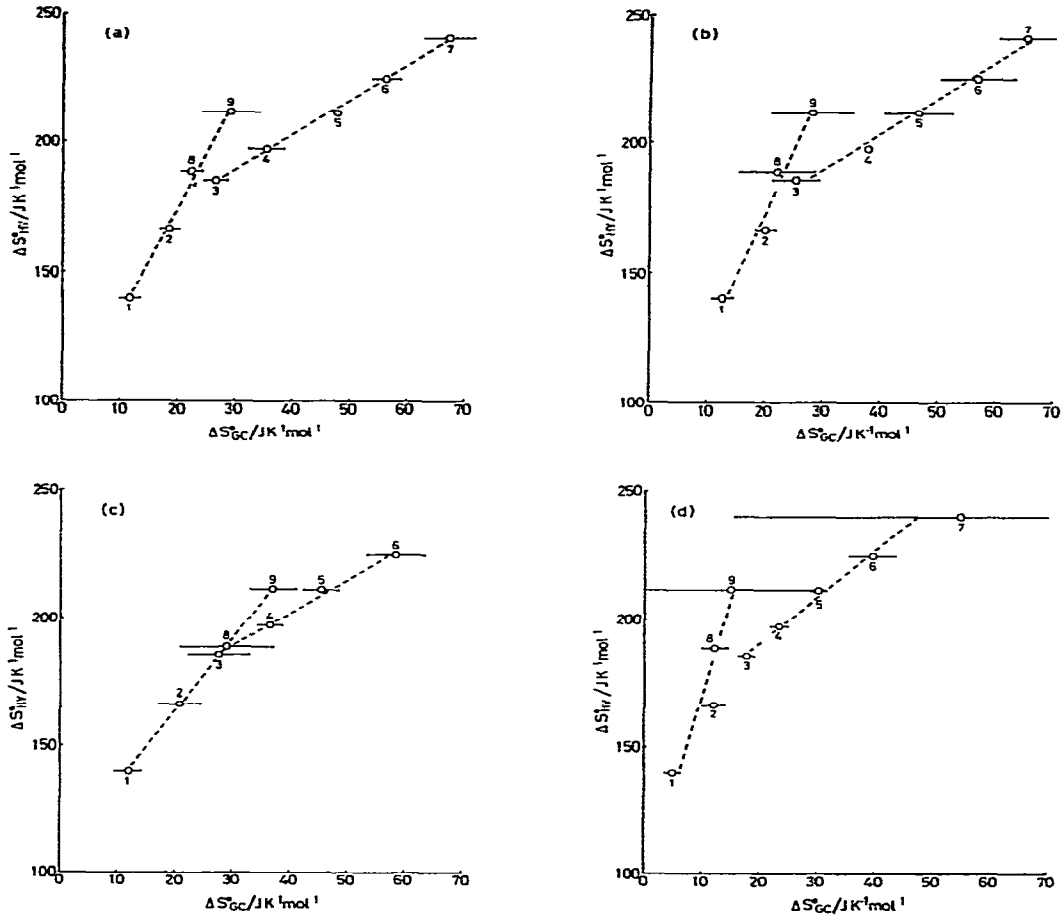

Fig. 5. Plots of ΔS°_{GC} against ΔS°_{HY}. Key as in Figs 3 and 4. ΔS°_{HY}: see text

permeation of the alcohol molecules into the swollen gel phase, where the solute will share the "hydrophobic hydration shells" in part with those of the gel matrix. The more carbon atoms there are in a molecule, the greater will be the entropy regain of the system. Consequently, $T\Delta S^{\circ}_{GC}$ terms overwhelm the opposite contribution of ΔH°_{GC} for several higher members of the primary *n*-alkanols. Fig. 6 illustrates schematically such a hydrophobic interaction mechanism between the solute and the gel matrix. The assignment of the hydrophobic site in the gel will be discussed later. A schematic energy diagram for hydrophobic solutes in the gaseous state, in aqueous solution and in the swollen gel phase is shown in Fig. 7. This is a possible outline of the hydrophobic interactions in gel chromatography.

There is reasonable evidence to explain the different tendencies of the "entropy regain" between groups A and B, phenomena corresponding to which were also observed on the enthalpy–entropy compensation behaviour in Fig. 4, as discussed in the preceding section. For aliphatic alcohols, Hill and White[16] observed that the correlation of ΔS°_{HY} with the surface area of the cavity in the water structure produced

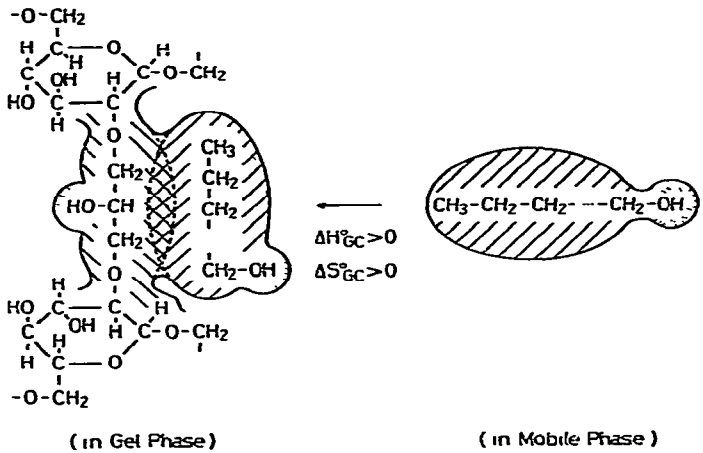

Fig 6. Schematic illustration of hydrophobic interaction in aqueous dextran gel system

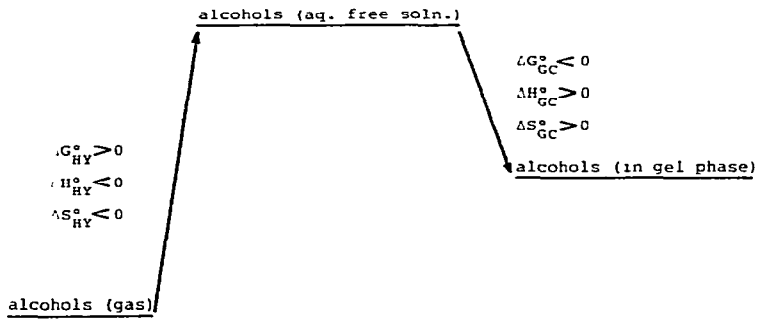

Fig 7. Schematic representation of energy diagram for alcohols as hydrophobic solutes in different states

by the dissolution of solute molecules in water for the members of group A is different from that for group B. Cabani and Gianni[27] found that the contribution of hydroxyl groups of saturated primary alcohols to the thermodynamic functions for hydration in water differs from that of secondary and tertiary hydroxyl groups. These facts may be attributable to different modes of hydration between groups A and B, as follows.

Group B consists of methanol and its derivatives in which the hydrogen atoms of methanol are replaced successively with methyl groups. Methyl substitution may influence the hydrophilic hydration shells on hydroxyl groups to a considerable extent. In contrast, the addition of methylene groups to a member of group A will not affect the hydrophilic hydration on the terminal hydroxyl groups. Therefore, the local dissimilarity of the mode of hydration between groups A and B may lead to different patterns of enthalpy–entropy compensation and of entropy regain. Strictly, the gel chromatographic behaviour of the aliphatic alcohols in aqueous systems, therefore, would not be described solely by the hydrophobic interaction mechanism, because the hydrophilic sites of the solutes and gel matrix might also participate in the separation mechanism. Molecular sieving may also be partly operative, especially for the members of group B.

In order to obtain additional information on the separation mechanism, the ΔS°_{GC} values of the alcohols are plotted against carbon number in Fig. 8, and the $K_{d(20)}$ and the thermodynamic functions for sodium and chloride ions in the eluent at pH 2 and 12 are listed in Table III.

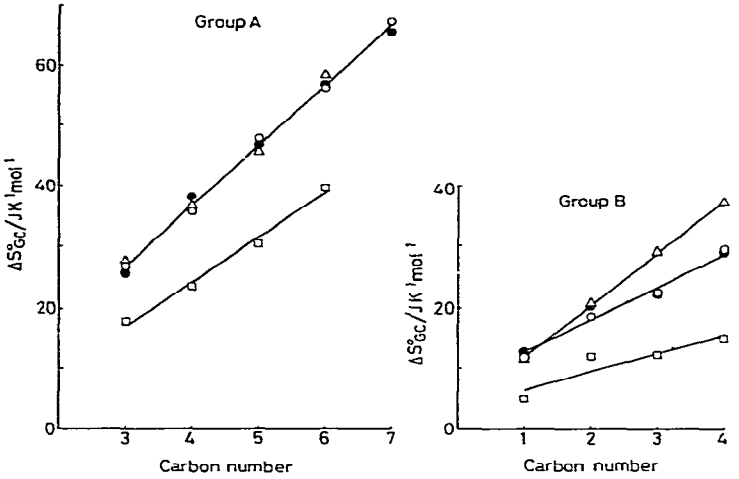

Fig 8 Plots of ΔS°_{GC} against carbon number. ○, on Sephadex G-10 with 0 1 *M* NaCl at pH 2; ●, on Sephadex G-10 with 0 1 *M* NaCl at pH 12; △, on Sephadex G-10 with water; □, on Sephadex G-15 with 0 1 *M* NaCl at pH 2.

TABLE III
$K_{d(20)}$ VALUES AND STANDARD THERMODYNAMIC FUNCTIONS FOR TRANSFER OF ELUENT IONS FROM MOBILE PHASE TO GEL PHASE
Gel, Sephadex G-10, eluent, 0 1 *M* NaCl at pH 2 and 12

Ion	*pH*	$K_{d(20)}$	ΔH°_{GC} (*kJ mol*$^{-1}$)	ΔS°_{GC} (*J*°*K*$^{-1}$ *mol*$^{-1}$)
Na^+	2	$0\,63_1$	−2.87 ± 1.50*	−13.6 ± 1 7*
Cl^-	2	0.65_4	−3 02 ± 1.02*	−13 8 ± 3 5*
Na^+	12	0.93_1	−6 39 ± 1 27*	−22 4 ± 4 3*
Cl^-	12	$0\,50_3$	−1.56 ± 0 97*	−11.0 ± 3.3*

* 0.95 confidence interval.

First, both ΔH°_{GC} and ΔS°_{GC} for the eluent ions are negative, in contrast to the positive values for the alcohols. The $K_{d(20)}$ value and the thermodynamic functions for sodium ion are nearly the same as those for chloride ion at pH 2, but they differ significantly at pH 12, indicating that at pH 12 sodium ion tends to be attracted and chloride ion to be repelled by the gel phase. This may be attributable to an increase in negative charge due to proton dissociation from hydroxyl groups fixed to the gel matrix, because the $K_{d(20)}$ value for sodium ion is the same as that for chloride at pH 12 on Sephadex LH-20 (hydroxypropyl derivative of G-25), according to preliminary experiments[28]. In contrast, the ln $K_{d(20)}$ values and the thermodynamic functions for each alcohol at pH 2 are almost same as those at pH 12 on Sephadex G-

10 with sodium chloride as eluent, as shown in Figs. 3 and 8. This behaviour is characteristic of the alcohols as non-electrolytes. In addition to the increase in negative charge of the gel matrix with increasing pH, the structure of water in the gel phase may change to some extent, depending on the pH of the eluent*. The possible change in water structure, however, could have a negligible influence on the separation mechanism of the alcohols, suggesting that the water structure around the hydrophobic sites of the gel matrix would scarcely alter with pH.

Secondly, Sephadex G-15 shows less affinity to the alcohols than the more tightly cross-linked gel Sephadex G-10, as can be seen in Fig. 8. Preliminary experiments also revealed that Sephadex LH-20 adsorbed the alcohols more than Sephadex G-15 did, in spite of its relatively large pore size[29]. These facts suggest that the hydrophobic sites of Sephadex G-10 and G-15 may be assigned to cross-links of the ether type. Several workers have also proposed that the ether-type linkages of dextran gels are responsible for hydrophobic adsorption[12,30–33]. As already shown, Fig. 6 reflects the fact that the solute molecules might interact hydrophobically with the cross-link of the gel matrix.

Thirdly, the K_d values of the eluent ions are considerably smaller than unity, except for that of sodium ion at pH 12, as shown in Table III. This fact provides the following possible interpretation of the effect of the eluent concentration on the K_d values in the higher concentration range at pH 2 (see Fig. 1). The smaller K_d values of the eluent ions suggest that there is a lower concentration of the salt in the gel phase than in the mobile phase. Sodium or chloride ion will change the water structures in the mobile and gel phases as a structure maker or breaker. The structure of water in the mobile phase will be perturbed more than that in the gel phase, because of the different solubilities of the salt between the two phases. The alcohol molecules in the bulk will therefore find it difficult to organize water, favorably around themselves in the higher concentration range of the eluent, so they might show affinity to the gel phase due to salting-out from the bulk. In other words, the different solubilities of the alcohols between the mobile and stationary phases due to the above cause may be responsible for the salting-out effect[1].

For group A, the salting-out effect was not observed at an eluent concentration of 0.1 M, as shown in Figs. 1 and 8. For *i*-PrOH and *t*-BuOH in group B, the ΔS°_{GC} and ln $K_{d(20)}$ values on Sephadex G-10 with water, however, are larger than those with sodium chloride eluents (see also Figs. 3 and 8). This phenomenon is contrary to that expected from the salting-out effect mentioned above, and might be attributable to the specific mode of hydration of group B, but this is not yet been clarified.

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* *Editor's Note* The paper by Y Yano and M. Janado [*J Chromatogr*, 200 (1980) 125] on the same topic had not appeared when this paper was submitted