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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF ALIPHATIC ALCOHOLS ON UNSUBSTITUTEDS EPHADEX GELS WITH HIGH DEXTRAN CONCENTRATIONS

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SUMMARY

An homologous series of *n*-aliphatic alcohols were found to be well separated by gel chromatography on a column of unsubstituted Sephadex G-10 in 2 *M* NaCl solution. The elution order of the alcohols was the reverse of that observed in gel permeation chromatography. The effects of temperature and salt concentration on K_{av} of the alcohols was in accord with hydrophobic interaction chromatography. The transfer free energy of the alcohols from external water to internal water of Sephadex G-10 became increasingly negative with increase in the number of carbon atoms of the hydrocarbon chain, *i.e.*, −330 cal per CH_2 group, and is entropic in nature. It is inferred that the hydrophobic solute is accommodated in the interstices formed in the cooperatively and radially oriented water confined in the condensed gel matrix, without substantial alteration, and perhaps with some enhancement, of the preexisting orientational order of the surrounding water molecules.

INTRODUCTION

In recent years, so-called hydrophobic interaction chromatography has been developed for the fractionation of proteins, using a series of alkyl-substituted agaroses as the adsorbent. In this method, protein separation is dependent on a whole set of variables including the hydrophobicity and density of the substituted alkyl groups, temperature, ionic strength and the hydrophobicity of the proteins. Multi-point attachment in the binding of proteins and large particles to the alkyl agaroses[1–3] may provide another separation parameter, but the detailed separation mechanism is not clearly understood[3–7]. Morris[7] suggested that the use of the term "hydrophobic interaction chromatography" be confined to the cases where all three of the following criteria are met: (1) hydrophobic sites can be identified on the stationary phase; (2) the solute is more firmly bound at higher rather than lower temperatures, so that the free energy of binding is primarily entropic in nature; (3) binding occurs at relatively high salt concentrations, and elution at lower concentrations. Haglund and Marsden[8] reported that non-polar compounds, *e.g.*, cyclohexane, are adsorbed on the more tightly crosslinked, but otherwise unsubstituted dextran (Sephadex) gels. Later, it

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was suggested that a hydrophobic interaction played an important rôle in this phenomenon, the non-polar faces of the anhydroglucose residues (Cl conformation) being considered to furnish the hydrophobic sites[9]. The anhydroglucose residue alone, however, seems to be insufficient to render the unsubstituted gels strongly hydrophobic, unless dextran molecules are so arranged as to produce a stable and regular array of the hydrophobic faces[7].

We have recently shown that not only Sephadex gels of high dextran concentrations (G-10, G-15) but also highly concentrated dextran solutions (>20%) are capable of dissolving a variety of hydrophobic solutes[10–12], and this seems to be best interpreted as being due to the anomalous nature of cooperatively hydrated water in the hydrophilic dextran matrix: the thermodynamic parameters pertaining to the transfer of the hydrophobic solutes from external water to internal water of Sephadex G-10 indicated that the "iceberg" formation is considerably diminished in hydrated water confined in the condensed gel matrix, thereby resulting in the relatively high solubilities of the hydrophobic solutes. In this communication, we show that a homologous series of *n*-aliphatic alcohols can be separated by hydrophobic interaction chromatography (HIC) using unsubstituted Sephadex G-10 as the adsorbent and 2.0 *M* aqueous NaCl solution as the eluent. On the basis of the thermodynamic data obtained, it is concluded that cooperatively hydrated water in the condensed gel matrix plays a crucial rôle in the present HIC system.

EXPERIMENTAL

Materials

n-Aliphatic alcohols (methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol and octanol) were purchased from Nakarai (Kyoto, Japan) and their purity was confirmed by gas chromatography before use. Sephadex gels and a dextran fraction (Dextran T500) were obtained from Pharmacia (Uppsala, Sweden) and Bio-Gel P-50 from Bio-Rad Labs. (Richmond, CA, U.S.A.). All other reagents used were of analytical reagent grade.

Gel chromatography

The alcohol mixtures for gel chromatography were prepared in 0.1 *M* NaCl solution. The final concentrations of methanol, ethanol and *n*-propanol in the mixtures were adjusted to 2%, and saturated solutions of *n*-butanol, *n*-pentanol, *n*-hexanol, *n*-heptanol and *n*-octanol were employed. Approximately 0.5 ml of the alcohol mixtures were applied on a temperature-controlled column of Sephadex gels pre-equilibrated with 0.1 *M* NaCl and eluted with 0.1 *M* NaCl at a rate of 15 ml/h using a constant-flow pump. In order to examine the effect of the medium composition, gel chromatography was also performed in 0.5 *M*, 1.0 *M* and 2.0 *M* NaCl solutions, urea solutions (2 *M*, 4 *M*, 6 *M* and 8 *M*) in 0.1 *M* NaCl, 50% aqueous ethylene glycol solution and 50% aqueous methanol solution.

Gas chromatography

The concentration of each alcohol in the collected fractions (0.5 ml) was determined by gas chromatography (GC) to produce the elution profiles. A gas chromatograph (Model GC-5A; Shimazuseisakusho, Kyoto, Japan) equipped with a

dual flame-ionization detector system and a digital integrator was employed. A glass column (4 m × 3 mm I.D.) packed with Shimalite TPA (Shimazuseisakusho) coated with 10% polyethylene glycol 6000 was used. For determination of alcohols in 0.1 *M* NaCl eluate, appropriate amounts of samples (0.6–2 μl) were directly injected into the gas chromatograph: injection temperature 150°C; column temperature 105°C for methanol, ethanol, *n*-propanol, *n*-butanol and *n*-pentanol, and 130°C for the higher alcohols; detector temperature 150°C; nitrogen flow-rate 60 ml/min; hydrogen flow-rate 50 ml/min; air flow-rate 1 l/min. The alcohols in other solvents (*i.e.*, 0.5–2.0 *M* NaCl solutions, 2–8 *M* urea solutions and 50% ethylene glycol solution) were extracted into suitable organic solvents (*e.g.*, *n*-butanol, *n*-heptanol, *n*-hexane) prior to gas chromatography.

Thermodynamic parameters

To estimate the dextran concentrations (C_f) of swollen Sephadex gels, the volume (V_G) occupied by the swollen gel beads (1 g dry weight) or the volume not available to non-penetrating solutes was determined by the equilibrium dilution experiment[13]. The values of C_f (g/100 ml) for Sephadex gels, calculated from $100 \times (1/V_G)$, were: 57.4 for G-10, 45.6 for G-15, 29.2 for G-25, 12.9 for G-50 and 5.8 for G-100. The apparent partition coefficients (K_{av}^{app}) of the alcohols on Sephadex gels were calculated from

$$K_{av}^{app} = (V_e - V_0)/(V_t - V_0) \quad (1)$$

where V_e is the elution volume, and V_t and V_0 the bed volume and void volume of the column, respectively.

In order to evaluate the thermodynamic parameters for the transfer of the alcohols from external water to internal water of the swollen gel phase, it is necessary to know the partition coefficient (K_{av}^0) pertaining to the ideal case of gel permeation chromatography were the chromatographic retention of the solute is determined solely by the steric exclusion effect and not by differential interaction of any sort[14]. The experimental determination of K_{av}^0 will be discussed later. The free energy change (ΔG^0) relevant to the transfer of the solute from external water to internal water can be obtained from

$$-\Delta G^0 = RT \ln K_{av}^{app}/K_{av}^0 \quad (2)$$

provided that interaction between the alcohols and the dextran chain of Sephadex gels is negligible[10,11]. The enthalpy change (ΔH^0) of the transfer process was evaluated from the van't Hoff plot and then the entropy change (ΔS^0) was obtained from the relation, $\Delta G^0 = \Delta H^0 - T\Delta S^0$ Since our primary interest is to investigate the differential properties of external and internal water, it may be more informative to include the contribution of the mixing entropy in ΔG^0 and, therefore, the thermodynamic parameters are given on molar concentration basis rather than on mole fraction basis.

RESULTS AND DISCUSSION

Effect of temperature and salt concentration on K_{av}^{app} of the alcohols for unsubstituted Sephadex gels

The values of K_{av}^{app} given in Table I were obtained with Sephadex G-10 in 0.1 *M* NaCl solution at various temperatures. K_{av}^{app} of the alcohols increases with increasing temperature, more markedly with increasing molecular weight. In contrast to gel permeation chromatography (GPC), the chromatographic retention increases with increase of molecular weight of the solutes. For Sephadex G-100, however, all the alcohols showed closely similar values of K_{av}^{app}, although there is some trend toward lower values of K_{av}^{app} with increase of the molecular weight (Table II). As is evident from Tables I and II, K_{av}^{app} of the higher alcohols markedly increases with increase of the dextran concentration (C_t) of Sephadex gels, and this is again in contrast with the case of GPC where K_{av} decreases with increasing concentration of the gel matrix.

TABLE I

K_{av}^{app} OF *n*-ALIPHATIC ALCOHOLS ON SEPHADEX G-10 AS A FUNCTION OF THE HYDROCARBON CHAIN LENGTH AND TEMPERATURE

The measurements were made in 0.1 *M* NaCl solution.

Alcohol	*25°C*	*30°C*	*35°C*	*40°C*
Methanol	0.67	0.66	0.67	0.69
Ethanol	0.67	0.67	0.69	0.74
n-Propanol	0.76	0.76	0.78	0.86
n-Butanol	0.92	0.97	1.00	1.07
n-Pentanol	1.25	1.33	1.31	1.45
n-Hexanol	1.67	1.75	1.93	2.05
n-Heptanol	2.45	2.58	2.81	3.04
n-Octanol	3.44	3.77	4.31	4.65

TABLE II

K_{av}^{app} OF *n*-ALIPHATIC ALCOHOLS ON SEPHADEX G-100

The measurements were made in 0.1 *M* NaCl solution at 20°C.

Alcohol	K_{av}^{app}	*Alcohol*	K_{av}^{app}
Methanol	0.66	*n*-Pentanol	0.65
Ethanol	0.66	*n*-Hexanol	0.63
n-Propanol	0.65	*n*-Heptanol	0.63
n-Butanol	0.65	*n*-Octanol	0.60

To examine the effect of salt concentration on K_{av}^{app} of the alcohols on Sephadex G-10, gel chromatography was performed in sodium chloride solutions of various concentrations (pH = 5.4 ± 0.07) at 20°C. The results for *n*-hexanol, *n*-heptanol and *n*-octanol are summarized in Table III which clearly shows that K_{av}^{app} increases appreciably with increasing salt concentration. The effect of the salt concentration on K_{av}^{app} of the lower alcohols was in the same direction as the higher alcohols, but

less striking in the salt concentration range (0.1–2.0 *M*) examined. Thus, the effects of temperature and salt concentration on K_{av}^{app} of the alcohols are both enhanced as the molecular weight of the solutes increases. Consequently, as shown in Fig. 1, the chromatographic resolution of the alcohols on Sephadex G-10 was greatly improved by increasing both temperature and salt concentration.

TABLE III

EFFECT OF THE SALT CONCENTRATION ON K_{av}^{app} OF THE ALCOHOLS ON SEPHADEX G-10

The pH of the sodium chloride solutions did not vary appreciably with the concentration (pH = 5.4 ± 0.07 at 20°C). All the measurements were made at 20°C.

Alcohol	K_{av}^{app}			
	0.1 M NaCl	*0.5 M NaCl*	*1.0 M NaCl*	*2.0 M NaCl*
n-Hexanol	1.57	1.65	nd	3.34
n-Heptanol	2.24	2.50	2.98	5.51
n-Octanol	3.23	3.86	4.88	9.60

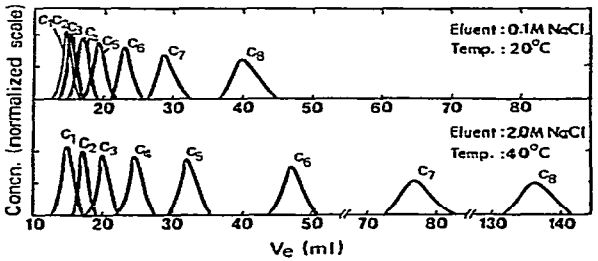

Fig. 1. Effect of temperature and salt concentration on the chromatographic resolution of *n*-aliphatic alcohols on unsubstituted Sephadex G-10. Peaks: C_1 = methanol; C_2 = ethanol; C_3 = *n*-propanol; C_4 = *n*-butanol; C_5 = *n*-pentanol; C_6 = *n*-hexanol; C_7 = *n*-heptanol; C_8 = *n*-octanol.

The mode of the dependence of K_{av}^{app} on salt concentration as well as the endothermicity of the partition process indicate that the chromatographic retention of the alcohols is primarily due to hydrophobic interaction. The present chromatographic system is different from the general type of hydrophobic interaction chromatography in that the gel used possesses no specific hydrophobic groups. However, as will be discussed later we consider, that cooperatively hydrated water confined in the gel matrices produces interstitial spaces, energetically favourable for accommodation of hydrophobic solutes, without substantial alteration of the preexisting orientational order of hydrated water.

Effect of urea, ethylene glycol and methanol on K_{av}^{app} of the alcohols

Inclusion of urea in the eluent (0.1 *M* NaCl) or elution with aqueous solutions of ethylene glycol or methanol led to decrease in K_{av}^{app} of the alcohols. The effect of urea was concentration dependent, and diminution of K_{av}^{app} of the alcohols with urea concentration became increasingly pronounced with increase of molecular weight.

However, even at 8 *M* the elution sequence of the alcohols remained unchanged (Table IV). Since this order is opposite to that expected for GPC, it follows that the interaction between the solutes and the swollen gel phase, presumably hydrophobic in nature, is not entirely abolished in 8 *M* urea solution. In 50% ethylene glycol solution, K_{av}^{app} of the alcohols decreases further, but as shown in Table IV the order of K_{av}^{app} is still not reversed.

TABLE IV

EFFECT OF ETHYLENE GLYCOL (EG), UREA AND METHANOL ON K_{av}^{app} OF THE ALCOHOLS ON SEPHADEX G-10

The values were obtained at 20°C.

Alcohol	K_{av}^{app}		
	50% EG	*8 M urea*	*50% methanol*
Methanol	0.67	0.68	0.67
Ethanol	0.67	0.64	0.64
n-Propanol	0.70	0.69	0.56
n-Butanol	0.78	0.77	0.45
n-Pentanol	0.94	0.80	0.34
n-Hexanol	1.33	1.11	0.24
n-Heptanol	1.72	1.25	0.22
n-Octanol	2.16	1.58	0.22

Since it is necessary, to know K_{av}^{0} of the alcohols corresponding to the ideal GPC, for evaluation of the thermodynamic parameters, an attempt was made to establish the condition under which the retention of the solutes is determined solely by the steric exclusion effect (or by the molecular sizes of the solutes). A 50% aqueous methanol solution was found to meet the above requirement: as shown in Table IV, the values and order of K_{av}^{app} of the alcohols conform to a GPC calibration expected for Sephadex G-10. Accordingly, the values obtained in 50% methanol solution (the last column of Table IV) may be safely taken as the K_{av}^{0} values of the respective alcohols. This is further justified by the following observations: first, at methanol concentrations above 40%, K_{av}^{app} of all the alcohols remained essentially constant; second, in 50% methanol solution, K_{av}^{app} of *n*-octanol, which exhibited a marked temperature dependence in the absence of methanol, was not appreciably affected by temperature. It is not yet possible to explain explicitly the effect of urea, ethylene glycol and methanol. However, as will be discussed later, we consider that these agents disrupt the orientational order of hydrated water in the condensed gel matrices, as well as the structure of free water, thereby nullifying or diminishing the preexisting differential properties of internal and external water arising from strong hydration.

Thermodynamic data for the transfer of the alcohols from external water to internal water of Sephadex G-10

The thermodynamic parameters for the transfer process were evaluated only for Sephadex G-10 on which the alcohols showed the highest K_{av}^{app} values. As previously discussed[10,12], ΔG^0 given by eqn. 2 refers to the free energy change for the passage of the solute from external water to internal water of the swollen gel only if

solute–gel fibre interaction is nil or negligible. To examine the interaction, the frontal gel chromatographic analysis was carried out on *n*-octanol (saturated)–Dextran T500 (50–200 m*M*) mixtures using a column of Bio-Gel P-50. (*n*-Octanol was chosen because it showed the highest K_{av}^{app} for Sephadex G-10.) The results indicated the absence of such an interaction between the two components. Accordingly, it follows that ΔG^0 of eqn. 2 refers to the free energy change arising solely from the differential interaction of the solute with external and internal water of the swollen gel.

In Fig. 2 ΔG^0 is plotted against the number of carbon atoms. As in the case of the transfer of a homologous series of alkanes from water to non-polar solvents[15], the linear dependence of ΔG^0 on the number of carbon atoms must reflect that ΔG^0 depends primarily on the magnitude of the hydrocarbon chain–water interfacial area. From Fig. 2 the increment of ΔG^0 per CH_2 group was found to be *ca.* −330 cal, which is, however, considerably less than that for the transfer from water to the alcohol itself[15]. Nevertheless, hydrated water confined in the condensed hydrophilic gel matrices seems to acquire organic solvent-like properties. It is of interest to note that a linear but positive dependence of ΔG^0 upon the number of carbon atoms generally pertains to GPC systems[16].

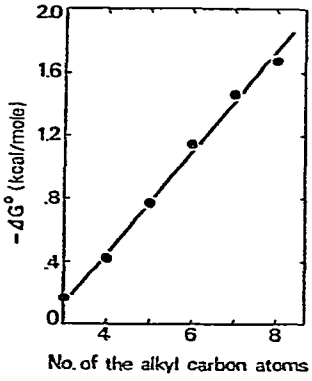

Fig. 2. Free energy for the transfer of the alcohols from external water to internal water of Sephadex G-10.

The enthalpy change (ΔH^0) was obtained from the van't Hoff plot (Fig. 3) and is given in Table V together with the entropy change (ΔS^0). Intrusion of the hydrophobic solutes in the internal water contained in the condensed gel matrix will possibly enhance the hydrophilic hydration of surrounding water molecules, leading to a decrease in enthalpy, but not to the extent of upsetting the overall endothermicity. The endothermicity of the transfer process may be attributed mainly to the extinction of the "iceberg" in external water. The driving force for the preferential partition of the alcohols for internal water of Sephadex G-10 is apparently entropic in nature, and may also be ascribable primarily to the extinction of the "iceberg" consequent upon the removal of the solutes from external free water. The above arguments led us to conceive an intuitive picture of the hydrocarbon chains dissolved in hydrated water: first, the solute molecule must be interposed in hydrated water confined in the condensed gel matrix, without substantial alteration of the preexisting orientational

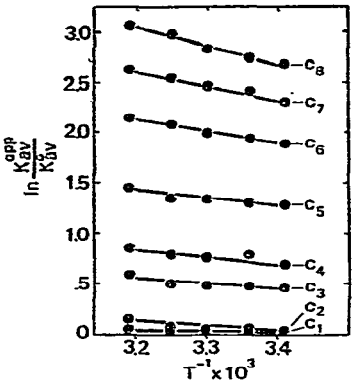

Fig. 3. Van 't Hoff plot for the transfer of the alcohols from external water to internal water of Sephadex G-10. Symbols C_1, C_2, C_3, etc., as in Fig. 1.

TABLE V

THERMODYNAMIC PARAMETERS OF TRANSFER OF THE ALCOHOLS FROM EXTERNAL WATER TO INTERNAL WATER OF SEPHADEX G-10 AT 25°C

Alcohol	ΔH^0 *(kcal/mol)*	ΔS^0 *(cal/K·mol)*
Methanol	0.00	0.00
Ethanol	0.33	1.20
n-Propanol	0.84	2.20
n-Butanol	1.32	5.87
n-Pentanol	1.78	8.59
n-Hexanol	2.31	11.61
n-Heptanol	2.73	13.96
n-Octanol	3.39	16.85

order of the surrounding hydrated water; second, the space occupied by the solute must have previously been occupied by free or more or less disordered water molecules which were expelled without great resistance by the intrusion of the solute; third, the degree of hydration or the orientational order of the surrounding hydrated water must be enhanced by the presence of the solute, thereby minimizing the interfacial contact. The validity of this tentative model will be assessed later in the light of additional information (see Fig. 5).

Effect of the dextran concentration (C_f) of Sephadex gels on K_{av}^{app} of n-octanol

The K_{av}^{app} value of *n*-octanol was found to increase exponentially with the dextran concentration (C_f) of Sephadex gels. Thus, the plot of K_{av}^{app} *vs.* C_f (Fig. 4) can be described by a regression curve of the 5th degree in C_f:

$$K_{av}^{app} = 1 + 3.35 \times 10^{-9}\, C_f^5 \qquad (3)$$

Various physical interpretations may be derived from this empirical relation. In a previous report[12] dealing with a similar formula, $K_{av}^{app} = 1 + kC_f^n$, pertaining to the SDS-Sephadex gel system, it was assumed that in the gel phase the solute (S) was

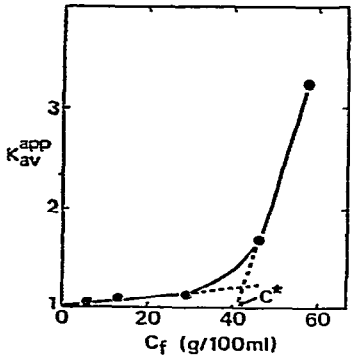

Fig. 4. Effect of the dextran concentration (C_f) of Sephadex gels on K_{av}^{app} of *n*-octanol. Solid line: regression curve based on $K_{av}^{app} = 1 + 3.53 \times 10^{-9} C_f^5$.

dissolved either in residual free water, as in the external phase, or in cooperatively hydrated water surrounded by *n* dextran chains (D). The solute in the latter state was regarded as an inclusion complex (SD_n). Considering a dynamic equilibrium between the two states ($S + nD \rightleftharpoons SD_n$), we derived an equation formally identical to the empirical formula, *i.e.*

$$K_{av}^{app} = 1 + K_{eq} C_f^n \tag{4}$$

where K_{eq} represents the equilibrium constant. By comparison of eqn. 4 and the empirical formula ($K_{av}^{app} = 1 + kC_f^n$), it was inferred that the empirical coefficient, *k*, referred to the equilibrium constant and the exponent *n* to the number of dextran chains surrounding hydrated water in which one solute molecule was accommodated.

As previously noted, however, this may not represent a unique interpretation of the experimental data. Indeed, if one assumes that the internal water undergoes a sharp physical change at a certain dextran concentration (*e.g.*, C^* in Fig. 4) as in the case of agar gel[17], the plot of K_{av}^{app} *vs.* C_f must be regarded as a composite of two (or more) independent lines (the broken lines). If this is the case, eqns. 3 and 4 are no longer valid. We are now dealing with two (or more) kinds of water with different solvent properties. Practically all the water molecules confined in the condensed hydrophilic gel matrix may be under the influence of highly polarized water firmly bound to the surrounding gel fibre and more or less immobilized, particularly in a highly condensed gel matrix due to the restricted thermal perturbation. The amount of such water, termed "cooperatively hydrated water", will increase rapidly as the dextran concentration of Sephadex gel is increased. The data presented in Fig. 4 are also explainable by assuming that the hydrophobic solutes are dissolved in the cooperatively hydrated water which increases exponentially with the dextran concentration of Sephadex gel. This contention is consistent with our observation[10–12] that a 40% solution of Dextran T500, but not of glucose, dissolved appreciable amounts of a variety of hydrophobic solutes including azobenzene, dimethylaminoazobenzene, alkylbenzenes and alkanes.

Description of the dissolved state of the hydrophobic solute in the internal water of Sephadex G-10

Suzuki *et al.*[17] measured the specific electric conductivity of agar gel as a function of the water content (C_w) of the gel and found that there were two sharp breaks in the plot at $C_w = 30\%$ and 55%. This was taken as evidence for the presence of at least three kinds of water in the concentrated agar gels. It was strongly supported by their subsequent NMR[18] and dilatometric[19] studies that revealed the presence of two kinds of water besides free water, *i.e.*, that which underwent the phase transition between 0°C and −20°C and which exhibited no transitional response in the range from −30°C to +20°C, presumably firmly bound water. The former type may be categorized under "cooperatively hydrated water". The amount of free water in the gel will decrease with increase of the gel fibre concentration (C_f), and the amount of firmly bound water will increase linearly or nearly linearly. The cooperative hydration, however, will be considerably enhanced in the condensed gel matrix owing to the restricted thermal motions of water molecules[11,12], and hence the amount of cooperatively hydrated water may be considered to increase steeply as C_f is increased. Thus, the parallelism between the relations of the solubility (K_{av}^{app}) of *n*-octanol (Fig. 4) and the amount of cooperatively hydrated water with C_f strongly suggests that it is the cooperatively hydrated water that is involved in the dissolution of the hydrophobic solutes.

For the sake of clarity, in the discussion that follows, diagrammatic models are presented illustrating the states of the internal water and of the dissolved solute. The models in Fig. 5, although based on the foregoing reasoning as well as on the results obtained from other studies[10–12], are by no means quantitative. Fig. 5a applies to a highly condensed gel matrix forming small pores in which practically all water molecules are either firmly bound (A) or cooperatively hydrated (B and B′). Distinction between B and B′ is made in Fig. 5a merely to indicate the decline of the orienting influence, imposed by firmly bound water, in the central region of the pore. Accordingly, the water molecules in this region will be less resistant to the intrusion of the hydrophobic solutes. Indeed, the energy required for the insertion of the solute molecule in this region may well be compensated by the subsequent enhancement of the cooperative hydration of the surrounding water, an exothermic process. The enhancement of the cooperative hydration may be justified by analogy with the "iceberg" formation[20] around the hydrophobic solute, and this is embodied in Fig. 5b. Since the overall transfer process is endothermic, the enthalpy change involved in the extinction of the "iceberg" in the external free water must be predominant in the

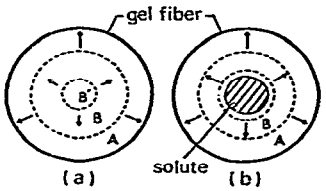

Fig. 5. Diagrammatic representation of the cooperative hydration of water confined in the condensed gel matrix (a), and accommodation of hydrophobic solute in the cooperatively hydrated water (b). The orienting tendency of water molecules due to hydrophilic hydration by the gel fibres is illustrated by a vectorial notation.

transfer of the alcohols. However, if the hydrophobicity of the solute is such that the "iceberg" formation occurs to a limited extent, as in the case of aromatic compounds[15], then the overall transfer process may be expected to be exothermic. Indeed, this was found to be the case with phenol and sulphanilamide[21,22]. As will be briefly noted later, the enthalpy change seems to be determined by the composite effect of hydrophobicity and the size of the solute relative to the pore size of the gel matrix.

In a low concentration gel matrix forming large pores, a large amount of free water will be left in the central region of the gel pore. As in the water structures around ions[23], three layers of water differing in the structural order may be formed in the pore, but their boundaries will be much obscured in this case. The low solubility of *n*-octanol in the low dextran concentration range (C_f) (Fig. 4) may be ascribed to the absence of cooperatively hydrated water. If, however, the size of the hydrophobic solute is of the same order as the volume occupied by free water in the gel pore, then a similar situation to that in Fig. 5b is expected to result. In fact, this postulate was partially substantiated by our finding of anomalous chromatographic behaviours of serum high density lipoprotein (HDL). That is, K_{av}^{app} of HDL for Sepharose 2B increased markedly with increasing salt concentration in the eluent and also with increasing temperature, indicating the operation of a mechanism similar to that pertaining to the alcohol–Sephadex G-10 system.

We have previously reported that inclusion of urea in the eluent led to decrease of K_{av} in gel chromatography of hydrophobic solutes on unsubstituted Sephadex gels and Bio-Gel[10–12,21,22], and this was explained as due to negation of the differential properties of the internal and external water by the overwhelming hydration effect of urea. As shown in Table IV, the presence of urea (8 *M*) and ethylene glycol (50%) diminished markedly the preferential partition of the alcohols in the internal water of Sephadex G-10, and in 50% methanol solution it was completely nullified. All these three agents, urea[24–26], ethylene glycol and methanol[27], are known to be so-called water structure breakers. Accordingly, it is conceivable that these agents destroy not only the structure of external water but also the orientational order of hydrated water, thereby depriving the internal water of its distinct properties. The effect of salt concentration in hydrophobic interaction chromatography has been a subject of controversy[6,7]. No additional conclusion can be drawn from the present study, but it seems that the effect of salt is two-fold, *i.e.*, decreasing the solubility of the hydrophobic solutes in the external water[15] and reducing the amount of free water in the gel phase, leading to accentuation of the preferential partition in the internal water phase. Solubility (mg/100 ml) of *n*-octanol was: 52.6 for water; 46.0 for 0.1 *M* NaCl; 23.0 for 1.0 *M* NaCl and 9.86 for 2.0 *M* NaCl.

Finally, we comment briefly on the studies reported by Okubo and Ise[28,29] on the solubility of hydrophobic solutes in macromolecular solutions. They found that solubilities of hydrophobic solutes (*e.g.*, naphthalene) are closely related to the overall hydrophobicity of the macromolecular solutes, *i.e.*, those promoting the "iceberg" formation (*e.g.*, polyvinylpyrrolidone) showed greater solvent capacity than the highly hydrophilic macromolecules (*e.g.*, starch, polyacrylamide). Accordingly, they concluded that the hydrophobic solutes were accommodated in the preformed or cooperatively formed "iceberg" structures around the hydrophobic segments of the macromolecules. It should be noted that their measurements were made in rather dilute macromolecular solutions. Thus, the solubilization mechanisms operating in

dilute and in highly condensed macromolecular solutions seem to be entirely different. As previously noted[10–12], highly concentrated dextran solutions are capable of dissolving appreciable amounts of a variety of hydrophobic solutes.

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