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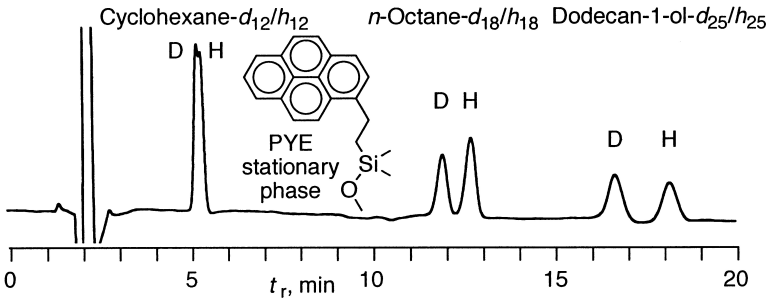

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Deuterium Isotope Effects on Hydrophobic Interactions: The Importance of Dispersion Interactions in the Hydrophobic Phase

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Abstract: Hydrogen/deuterium isotope effects on hydrophobic binding were examined by means of reversed-phase chromatographic separation of protiated and deuterated isotopologue pairs for a set of 10 nonpolar and low-polarity compounds with 10 stationary phases having alkyl and aryl groups bonded to the silica surface. It was found that protiated compounds bind to nonpolar moieties attached to silica more strongly than deuterated ones, demonstrating that the CH/CD bonds of the solutes are weakened or have less restricted motions when bound in the stationary phase compared with the aqueous solvent (mobile phase). The interactions responsible for binding have been further characterized by studies of the effects of changes in mobile phase composition, temperature dependence of binding, and QSRR (quantitative structure−chromatographic retention relationship) analysis, demonstrating the importance of enthalpic effects in binding and differentiation between the isotopologues. To explain our results showing the active role of the hydrophobic (stationary) phase we propose a plausible model that includes specific contributions from aromatic edge-to-face attractive interactions and attractive interactions of aliphatic groups with the π clouds of aromatic groups present as the solute or in the stationary phase.

Introduction

Reversed-phase, high-performance liquid chromatography (RPLC) involves transfer of solute from a polar, aqueous mobile phase to a nonpolar, hydrophobic stationary phase. Partitioning between mobile and stationary phase has been shown to be the predominant retention mechanism, and thus RPLC constitutes a useful model of hydrophobic effects that are of great importance in biology and chemistry. The opportunity exists to capitalize on the very high precision of RPLC to measure isotope effects, which constitute a unique probe of molecular interactions, upon transfer of hydrophobic molecules from an aqueous to a nonpolar phase.

We have previously shown the utility of RPLC for highly precise isotope effect studies.[1−4] Advances in understanding of hydrophobic effects in general and, simultaneously, of the RPLC separation process should be accessible through further study of RPLC isotope effects over a wider range of stationary phase structures, solute structures, and mobile-phase compositions.

Hydrophobic interactions play a key role in determining the structure and function of lipid membranes[5] and proteins[6] as well as the activity of the drugs and toxins.[7,8] Several techniques for

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evaluation of hydrophobic properties are available, including measurement of *n*-octanol/water partition coefficients *P* for a series of compounds.[9] RPLC[10,11] methods have provided an excellent method to give reliable hydrophobicity measurements.[12]

H/D isotope effects are widely used to characterize chemical processes.[13−16] RPLC separations of protiated and deuterated pairs of compounds[1,4] and separation of nitrogen and oxygen isotopes in acids and bases[2] have been reported. Tritium isotope effects in high-performance liquid chromatography (HPLC) of eicosanoids and vitamin D metabolites have also been reported.[17,18] Recently, separation of enantiomers based on *isotopic chirality* was described.[3] A cellulose-based stationary phase was shown to separate racemic phenyl(phenyl-d_5)methanol, thus demonstrating that the chiral stationary phase exhibits different interactions with the two enantiomers even though they differ only by the stereochemical positioning of phenyl and phenyl-d_5. These results show that liquid chromatography techniques constitute highly convenient and sensitive tools for precise study of isotope effects.

In this work we have extensively examined secondary isotope effects in the retention process of RPLC, as a means of investigating the nature of hydrophobic effects. In addition, both HPLC and mass spectrometric (MS) methods such as isotope-coded affinity are used to compare protein profiles of different cells, e.g., diseased vs healthy.[19,20] The ability to separate isotopes by HPLC suggests the possibility of combining current LC-MS methods with isotope-coded differential LC to provide a new method for measurement of differential protein content.

The nature of isotope effects involves to a large extent vibrational frequencies.[21] Within the commonly accepted Born−Oppenheimer approximation, the electronic wave functions and the resulting potential energy surfaces do not change upon isotopic substitution but the vibrational wave functions of deuterium and protium are different because the vibrational states depend on the mass of the nuclei. Ordinarily, structural changes in solute, solvent, or stationary phase will involve changes in potential energies of interaction which are very difficult to estimate theoretically, so that interpretation of binding effects is also very difficult. But since isotopic substitution does not affect potential energy, potential energy effects cancel between isotopologues, and isotope effects involve only the effects of changes in interactions upon nuclear motions, especially upon vibrational frequencies. Consequently, isotope effects probe changes in molecular interactions without involving the difficulties of interpreting potential energy changes. Isotope effects therefore provide a unique and readily interpreted experimental means of investigating molecular interactions.

As a result of its larger mass, the amplitude of vibrations is smaller for deuterium, also resulting in slightly lower average volumes and polarizabilities for bonds involving deuterium than for the corresponding bonds involving protium.[22] We use calculations of these effects to give additional insights into the isotope effects we have observed.

Measuring retention factors in RPLC gives direct information about the free energy associated with transfer of a nonpolar solute from an aqueous mobile phase to an organic stationary phase, corresponding to the binding process, and the effect of isotopic substitution on this process (cf. eq 1−5 below). Though a complete description of the hydrophobic binding process is still under development,[5] fundamental work has related hydrophobic effects to retention mechanisms in liquid chromatography.[23−27] RPLC involves an equilibrated aqueous mobile phase and an organic stationary phase, the partitioning process being controlled by both mobile-phase and stationary-phase effects. Mutual van der Waals interactions between the nonpolar stationary phase and solutes operate, in particular, dispersion forces, resulting in attractive binding of solute within the stationary phase. Because of the unique ability of isotope effects to probe the nature of solute interactions, our studies allow us to dissect the total hydrophobic process into contributions from mobile phase hydrophobic phenomena and from binding properties of the stationary phase.

Simulation techniques, such as the free energy perturbation method with commonly used molecular dynamics force fields,[28] are usually unable to reproduce the experimental numbers with satisfactory accuracy for the purposes of analyzing HPLC retention processes. Because such difficulties are inherent in accurately estimating small changes in interaction energies, we employ here quantitative structure−chromatographic retention relationships[11] (QSRR) analysis, an extrathermodynamic linear free energy relationship (LFER), to aid in elucidating the nature of hydrophobic binding in RPLC.[29]

The stationary phases tend to resemble organic liquid phases but differ in that they possess significant ordering resulting from the attachment of the hydrophobic chains to the silica particle core.[25] These stationary phases will bind the methanol component of the mobile phase to some extent, so the properties we measure refer to a methanol-saturated stationary phase. Almost

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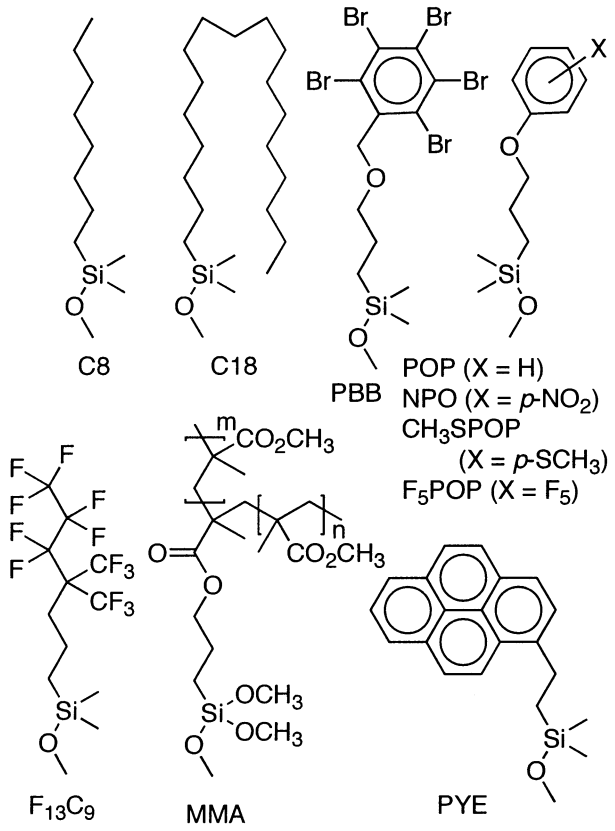

Figure 1. Structures of the stationary phases used.

all of the solutes we have chosen for study must interact with the stationary phase almost entirely, if not exclusively, by partitioning into the hydrophobic layer. Interaction with the silica particle core is believed to be minimal and most probably negligible for several reasons. First, the stationary phases are prepared with a high density of covalently attached organic chains that largely mask any residual, underivatized SiOH sites. Second, the isotopically substituted solutes studied are mainly nonpolar hydrocarbons. Third, our data show nearly identical values of the isotope effect per CH/CD bond for aliphatic alcohols as for aliphatic hydrocarbons (though differing among different stationary phases), indicating that the hydrocarbon parts of the chains are in closely similar environments regardless of the presence or absence of an attached alcohol group. Finally, our QSRR analysis shows that binding to all of these stationary phases is primarily if not entirely hydrophobic in nature. Thus the stationary phases we have studied have a hydrophobic solute-binding mechanism.

Our isotope effect data do not of course provide information about the exact structure or ordering of the stationary phases and the bound solutes, but our data do tell us about the nature and extent of solute−stationary phase interactions.

Experimental Section

Equipment. The following analytical equipment was used for the chromatographic experiments: an LC-10AD pump at a flow rate 1 mL min^{-1} and SPD-10AV UV−Vis detector from Shimadzu (http://www.shimadzu.com/) at $\lambda = 254$ nm for the aromatic compounds and a JASCO 830-RI refractive index detector for aliphatic compounds (http://www.jasco.co.jp/). Constant column temperature was maintained with a water bath. Chromatograms (using mobile phases CH_3OH/H_2O, CH_3CN/H_2O) were collected and analyzed by a V-STATION chromatography data system (http://www.gls.co.jp/) on a PC.

Materials and Methods. Deuterated compounds were available from Aldrich or CEA (Commisariat à L'Energie Atomique, France). 1-Decan-d_{21}-ol was prepared from decanoic-d_{19} acid, 1-pentan-d_{11}-ol was prepared from pentanoic-d_9 acid, and phenyl-d_5-methyl alcohol was prepared from the benzoic-d_5 acid. All other chemicals were of analytical grade and were available from the common major suppliers.

We employed a variety of structures for the stationary phase materials (Figure 1), all based on silica bonded beads: aliphatic octyl C8 and octadecyl C18, fluorinated 4,4-di(trifluoromethyl)-5,5,6,6,7,7,7-heptafluoroheptyl, $F_{13}C_9$, highly dispersive 3-(pentabromobenzyloxy)propyl, PBB, highly aromatic 2-(1-pyrenyl)ethyl, PYE, aromatic 3-(*p*-nitrophenoxy)propyl, NPO, 3-phenoxypropyl, POP, 3-(*p*-methylmercaptophenoxy)propyl, CH_3SPOP, 3-(pentafluorophenoxy)propyl, F_5POP, and polymer-bonded silica/methyl methacrylate, MMA. C8, C18, POP, CH_3SPOP, F_5POP, and MMA were prepared in our laboratory, according to procedures described elsewhere.[30,31] $F_{13}C_9$ was commercially available from Wako Pure Chemical Industries, Ltd. (http://www.wako-chem.co.jp); PBB, PYE, and NPO were available from Nacalai Tesque (http://www.nacalai.co.jp/).

Determination of the Retention Factors and Isotope Effects. The partition coefficient in chromatography is expressed as a retention factor:

$$k = (t_r - t_0)/t_0$$

where t_r is the retention time of a solute on a given column and t_0 is the retention time of a nonretained solute ("dead time"). The retention factor relates to free energy changes according to eq 1

$$\ln K = -\Delta G^0/RT = -\Delta H^0/RT + \Delta S^0/R = \ln k - \ln \phi \quad (1)$$

where K is the chromatographic binding equilibrium constant and ϕ is the column phase ratio, i.e., the ratio of the volumes of the stationary and the mobile phases. The retention factor of a methylene unit α-(CH_2), commonly used in chromatography, was also calculated. It is the ratio of the retention factors for two homologues, for example,

$$\alpha(CH_2) = k_{\text{1-pentanol-}h_{11}}/k_{\text{1-butanol-}h_9} \quad \text{and}$$
$$\alpha(CD_2) = k_{\text{1-pentanol-}d_{11}}/k_{\text{1-butanol-}d_9} \quad (2)$$

for the protiated and deuterated homologues, respectively. In addition, we measured $\alpha(CH_2)$ using amylbenzene and butylbenzene. We have introduced in this work another unit that reflects the retention behavior of aromatic compounds

$$\alpha(C_4H_2) = k_{\text{naphthalene-}h_8}/k_{\text{benzene-}h_6} \quad (3)$$

which we term the "retention factor of an aromatic unit".

The total isotope effect (TIE) is calculated to reflect the overall difference in chromatographic behavior of each isotopologue pair. It is free from any phase ratio influence (cf. eq 1, ϕ cancels out), since it is calculated as a ratio

$$\text{TIE} = k_H/k_D$$

where k_H and k_D are the retention factors for the protiated and deuterated isotopologue pair, respectively. Therefore, one may calculate direct free energy values for this process as well. The single isotope effect (%IE) reflects the average influence of a single H/D substitution and is given by

$$\%\text{IE} = 100[(k_H/k_D)^{1/n} - 1] \quad (4)$$

where n is the number of D atoms substituted for H.

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Table 1. Dependence of Retention Capacity Factors, *k*, Isotope Effects TIE[a] (Total) and %IE[b] (Single), and Structural Unit Retention Factors α on Structure of Stationary Phase[c]

compound	C8	C18	PYE	POP	NPO	F_5POP	$F_{13}C_9$	CH_3SPOP	PBB	MMA
benzene	0.872	1.507	0.850	0.695	0.514	0.801	0.420	0.980	1.393	0.704
benzene-d_6	0.847	1.455	0.825	0.679	0.505	0.791	0.417	0.956	1.343	0.698
TIE	1.030	1.036	1.030	1.024	1.018	1.013	1.007	1.025	1.037	1.008
%IE	0.486	0.586	0.499	0.390	0.294	0.209	0.120	0.414	0.611	0.143
toluene	1.358	2.609	1.342	0.977	0.733	1.250	0.564	1.387	2.426	0.890
toluene-d_8	1.310	2.498	1.286	0.949	0.716	1.233	0.562	1.343	2.316	0.881
TIE	1.037	1.044	1.044	1.030	1.024	1.014	1.004	1.033	1.047	1.011
%IE	0.450	0.545	0.534	0.364	0.293	0.171	0.045	0.404	0.582	0.127
naphthalene	1.768	3.847	2.745	1.624	1.470	2.045	0.451	2.581	7.299	1.809
naphthalene-d_8	1.696	3.644	2.621	1.575	1.435	2.003	0.445	2.493	6.909	1.796
TIE	1.042	1.056	1.047	1.031	1.024	1.021	1.013	1.035	1.056	1.007
%IE	0.522	0.680	0.579	0.384	0.302	0.260	0.168	0.435	0.688	0.090
anthracene	3.727	11.595	11.629	3.978	4.890	5.168	0.511	8.087	46.854	4.172
anthracene-d_{10}	3.532	10.762	10.895	3.829	4.737	5.020	0.502	7.724	43.472	4.145
TIE	1.055	1.077	1.067	1.039	1.032	1.029	1.018	1.047	1.078	1.007
%IE	0.539	0.748	0.654	0.382	0.318	0.291	0.178	0.460	0.752	0.065
nitrobenzene	0.629	0.887	2.016	0.835	0.950	0.952	0.379	1.292	1.897	1.032
nitrobenzene-d_5	0.617	0.865	1.941	0.818	0.936	0.946	0.379	1.263	1.841	1.026
TIE	1.019	1.025	1.039	1.021	1.015	1.006	1.000	1.023	1.030	1.006
%IE	0.385	0.503	0.760	0.413	0.298	0.126	0.000	0.456	0.601	0.116
cyclohexane	3.481	7.836	1.986	1.599	1.010	1.695	1.348	1.937	3.101	0.951
cyclohexane-d_{12}	3.373	7.519	1.901	1.553	0.988	1.689	1.369	1.869	2.955	0.939
TIE	1.032	1.042	1.045	1.030	1.022	1.004	0.985	1.036	1.049	1.012
%IE	0.263	0.345	0.365	0.243	0.184	0.030	−0.128	0.298	0.403	0.106
hexane	5.055	12.136	2.608	1.856	1.166	2.510	2.226	2.224	3.840	0.971
hexane-d_{14}	4.901	11.565	2.468	1.795	1.136	2.502	2.255	2.130	3.619	0.956
TIE	1.031	1.049	1.057	1.034	1.026	1.003	0.987	1.044	1.061	1.015
%IE	0.221	0.345	0.395	0.239	0.186	0.023	−0.093	0.309	0.425	0.111
octane	11.887	35.406	6.320	3.487	2.119	5.063	3.968	4.492	10.014	1.630
octane-d_{18}	11.387	33.264	5.878	3.334	2.044	5.022	4.061	4.235	9.275	1.599
TIE	1.044	1.064	1.075	1.046	1.037	1.008	0.977	1.061	1.080	1.019
%IE	0.239	0.347	0.404	0.250	0.200	0.045	−0.129	0.328	0.427	0.107
1-decanol	5.278	10.292	4.307	2.166	1.349	2.508	1.843	2.680	5.580	0.693
1-decanol-d_{21}	5.009	9.588	3.963	2.062	1.297	2.485	1.886	2.517	5.118	0.672
TIE	1.054	1.073	1.087	1.050	1.040	1.009	0.977	1.065	1.090	1.032
%IE	0.249	0.338	0.397	0.234	0.187	0.044	−0.110	0.299	0.413	0.147
1-dodecanol	12.036	29.850	9.573	3.991	2.390	4.907	3.248	5.266	13.930	1.167
1-dodecanol-d_{25}	11.312	27.400	8.680	3.764	2.274	4.862	3.345	4.882	12.563	1.125
TIE	1.064	1.089	1.103	1.060	1.051	1.009	0.971	1.079	1.109	1.038
%IE	0.248	0.343	0.393	0.234	0.199	0.037	−0.118	0.303	0.414	0.147
α(CH_2)	1.510	1.680	1.540	1.380	1.350	1.380	1.330	1.410	1.590	1.296
α(C_4H_2)	2.028	2.553	3.229	2.337	2.860	2.553	1.074	2.634	5.240	2.570
n_D^{30}[d]	1.404	1.441		1.513	1.576	1.427	1.295[e]	1.573		

[a] TIE = k_H/k_D. [b] %IE = $100[(k_H/k_D)^{1/n} - 1]$. [c] Cf. Figure 1; 70% methanol/water (vol/vol) mobile phase, 30 °C. [d] Experimentally measured refractive indices for the olefins used as the precursors for the stationary phases. [e] Measured at 20 °C.

Molecular Modeling and QSRR Calculations. Molecular modeling and structural descriptor calculations were done using HyperChem 5.1 Pro with ChemPlus 1.5 (http://www.hyper.com/). Protiated structures were first calculated by the extended Hückel method in order to estimate charges on the atoms and then optimized with the MM+ force field followed by RHF semiempirical (AM1) geometry optimization in a vacuum. The next step was placing the AM1 optimized structures inside a periodic box of water molecules and another optimization of geometry, where water was treated by a MM+ force field (classical approach) and the solute was treated by AM1 (semiempirical approach). For these optimized solute structures, calculations of the van der Waals surfaces and volumes were performed using the appropriate van der Waals radii values.

Quantitative structure−chromatographic retention analyses by means of simple and multiple linear regression were performed on a PC machine using Statlets 1.1B (http://www.statlets.com/) and Prophet 5.0 (http://www.bbn.com/), freely available on the web for research purposes.

Results

This work investigates structural effects upon chromatographic binding equilibria, that is, equilibria for partitioning of solutes between aqueous and hydrophobic phases. By studying the effects of changes in aqueous phase composition for different hydrophobic phases, of changes in hydrophobic phase for different aqueous phase compositions, and of different solutes as a function of both aqueous phase and hydrophobic phase composition, we are able to dissect important factors that contribute to the phenomenon of hydrophobic binding. To this end, we give below our results for (a) isotope effects upon binding, (b) linear free energy relationships, (c) structural unit retention factors α(CH_2) for methylene and α(C_4H_2) for aromatics, and (d) temperature effects.

Retention Factors. The retention data along with the total and single isotope values (TIE and %IE), for 10 isotopologue pairs on all stationary phases in 70% methanol/water mobile phase are given in Table 1. Results for the other mobile phase compositions are given in the supporting information (Table A). Samples injected were sufficiently small (ca. 10−20 μg aliphatic, ca. 1−2 μg aromatic) to approach infinite dilution in the mobile phase and also permit solubility in the aqueous mobile phases employed. The resulting chromatograms show minimal distortions in peak symmetry that might result from saturation of binding sites in the stationary phase. Representative chromatograms are shown in Figure 2.

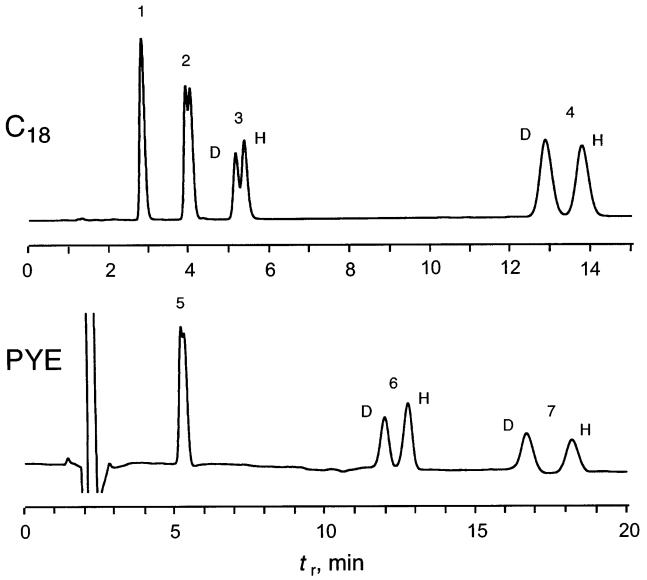

Figure 2. Representative chromatograms of H/D separation in a 70% methanol/water mobile phase for seven isotopologue pairs on C18 and PYE phases. 1, Benzene-h_6/d_6; 2, toluene-h_8/d_8; 3, naphthalene-h_8/d_8; 4, anthracene-h_{10}/d_{10}; 5, cyclohexane-h_{12}/d_{12}; 6, *n*-octane-h_{18}/d_{18}; 7, dodecan-1-ol-h_{25}/d_{25}.

The largest retention factors for aromatic solutes were exhibited by the aromatic PBB (pentabromobenzyloxypropyl) and PYE (pyrene) stationary phases. High *k* values are observed with the octadecyl phase (C18) for aliphatic compounds. Although the same mobile phase was used in each case, the capacity factors *k* still do not directly provide the actual binding equilibrium constants *K* and free energies, since every column differs according to its phase ratio ϕ (eq 1). Because ϕ is difficult to determine, accurate calculation of thermodynamic parameters remains problematic.

Cancellation of ϕ in Ratio-Based Structural Unit Retention Factors and Isotope Effects. Direct calculation of binding free energy becomes possible by defining structural unit retention factors $\alpha(CH_2)$ for methylene and $\alpha(C_4H_2)$ for aromatics, which are computed from the retention data (eqs 2 and 3). Since (eq 1) $K = k/\phi$, the difficult-to-determine phase ratio ϕ cancels when ratios of *k* values are taken, with the result that the α factors equal true ratios of equilibrium constants *K*, as shown in eq 5. For the same reason, ϕ cancels out in isotope effects, so that the ratio of retention factors k_H/k_D equals K_H/K_D, the true equilibrium isotope effect on binding.

$$k_1/k_2 = K_1\phi/K_2\phi = K_1/K_2 \quad \text{and}$$
$$k_H/k_D = K_H\phi/K_D\phi = K_H/K_D \quad (5)$$

For the same mobile phase composition, differences observed reflect the diverse properties and behavior of the stationary phases. In particular, we observed a similar order of elution of homologues upon all phases, while the H/D-pairs' elution order was reversed in certain cases.

Characterization of Stationary Phase Properties by Means of QSRR. Quantitative structure−chromatographic retention relationship (QSRR) analysis of retention data has an extrathermodynamic character[32] and is useful in investigating molecular mechanisms of retention, such as hydrophobic binding,[33] as well as in retention prediction.[34] To provide a direct comparison of stationary phases, we carried out QSRR analysis for all 10 stationary phases. We employed the same series of 33 test solutes, under the same conditions, for each (see Table B in the supporting information) and used the structural descriptors proposed by Abraham[35] to characterize the stationary phase properties.[36] Abraham's equation (eq 6), derived from the Kamlet−Taft solvatochromic parameters,[37]

$$\log k = m + \nu V_x + S\pi_2^H + a\Sigma\alpha_2^H + b\Sigma\beta_2^H + \rho R_2 \quad (6)$$

describes retention in terms of molecular properties of solutes and the chromatographic mobile and stationary phases, where *m* is a constant (intercept), V_x is the McGowan characteristic volume of the solute, α_2^H is the hydrogen-bond acidity of the solute, β_2^H is the hydrogen-bond basicity of the solute, π_2^H is the dipolarity−polarizability of the solute, and R_2 is the excess molar refraction of the solute. Respective coefficients ν, *S*, *a*, *b*, and ρ reflect differences in properties between the stationary and mobile phases, to be discussed later. Table 2 presents the results of multiple regression analysis of retention data with 70% methanol/water mobile phase composition. The coefficients are related to the properties of both the stationary and mobile phases. However, if the same mobile phase is employed with different stationary phases, as in Table 2, the mobile phase properties cancel out in comparing the coefficients for different stationary phases. The results are presented for independent variables giving a significance level, *p*, ≤ 0.05. QSRR regression

Table 2. Results of QSRR Analysis (Eq 6) of 33 Test Compounds for a Series of Stationary Phases[a]

stationary phase SD[b]	*m*	ρ	*S*	*a*	*b*	*n*	$R^{2\,c}$ (*p*)
C8	−0.730	[d]	−0.364	−0.208	−1.304	1.393	0.987
SD	0.060		0.036	0.032	0.056	0.059	($\leq 10^{-4}$)
C18	−0.584	0.242	−0.679	−0.298	−1.509	1.632	0.991
SD	0.066	0.077	0.066	0.036	0.079	0.071	($\leq 10^{-4}$)
PYE	−1.218	[d]	0.498	−0.848	−1.154	1.428	0.963
SD	0.107		0.065	0.057	0.099	0.105	($\leq 10^{-4}$)
POP	−0.843	[d]	[d]	−0.371	−1.025	1.124	0.986
SD	0.046			0.023	0.041	0.047	($\leq 10^{-4}$)
NPO	−1.070	[d]	0.327	−0.330	−1.167	1.053	0.959
SD	0.077		0.046	0.040	0.071	0.075	($\leq 10^{-4}$)
F_5POP	−0.740	−0.139	[d]	−0.276	−1.130	1.218	0.954
SD	0.088	0.067		0.049	0.078	0.093	($\leq 10^{-4}$)
$F_{13}C_9$	−0.677	−0.433	[d]	−0.438	−1.033	0.981	0.964
SD	0.084	0.061		0.047	0.075	0.089	($\leq 10^{-4}$)
CH_3SPOP	−0.904	0.166	[d]	−0.419	−1.084	1.175	0.980
SD	0.058	0.042		0.032	0.051	0.061	($\leq 10^{-4}$)
PBB	−0.915	0.236	[d]	−0.476	−1.352	1.520	0.975
SD	0.076	0.054		0.042	0.067	0.080	($\leq 10^{-4}$)
MMA	−0.710	[d]	0.253	−0.143	−1.490	0.859	0.948
SD	0.078		0.058	0.044	0.090	0.071	($\leq 10^{-4}$)

[a] Cf. Figure 1; mobile phase 70% methanol/water, 30 °C. [b] SD = standard deviation of the coefficient. [c] R^2 = determination coefficient of the multiple regression analysis; *p* = significance level of the equation (eq 6). [d] Not included in correlation because significance level *p* was higher than 0.05.

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analysis was also performed including all variables regardless their significance levels (see Table C in the supporting information). Inclusion of all variables gives trends similar to those shown in Table 2. The coefficients indicate the sensitivity of solute binding to changes in stationary phase (while keeping the mobile phase constant at 70% methanol/water). For all stationary-phase materials,[30] coefficients *a* (sensitivity to solute hydrogen bond acidity) and *b* (sensitivity to solute hydrogen bond basicity) are negative, while *v* (sensitivity to solute McGowan characteristic volume) is always positive. Coefficient *S* (sensitivity to solute dipolarity-polarizability) is positive for PYE, NPO, and MMA but negative for C8 and C18 stationary phases, while ρ (sensitivity to solute excess molar refraction) is negative only for the fluorinated stationary phases.

Table 1 includes the values of the available refractive indices of the precursors of the stationary phases. The refractive index is directly related to the molecular polarizability of compounds, according to the Lorentz−Lorentz formula (eq 7). The polarizability properties of the stationary phases may be qualitatively

$$\alpha = (n_r^2 - 1)/(n_r^2 + 2) \qquad (7)$$

compared using these n_D[30] values to approximate the relative values for the chains of the stationary phase. Although the refractive indices of those expected to have the highest values, PBB and PYE, are not available, one notes that the precursors of the remaining aromatic phases have the greatest values, followed by the aliphatic C18 and C8. The precursor of the $F_{13}C_9$ phase has a refractive index smaller than that for water ($n_D^{30} = 1.333$).

The negative coefficients *a* and *b* reflect the fact that polar interactions of solutes are much stronger in the aqueous mobile phase than in the stationary phase. The fact that *a* and *b* for all stationary phases are similar to that for the C18 phase indicates that solute binding by all phases is almost exclusively of a hydrophobic nature, especially involving London dispersion attractive interactions, as is already widely accepted for C18 stationary phases.[36] That this similarity exists even for the NPO phase, which has a *p*-nitrophenyl group, indicates that the nitro groups are probably at the surface of this phase and interacting primarily with the aqueous mobile phase and that even in this case the binding of solutes is hydrophobic in nature. The data thus indicate that interactions, even of polar solutes, with the silica core of these stationary phases are most probably negligible. With our nonpolar solutes, if any interactions involving the silica core were present, they would most probably be with the polar methanol component of the aqueous phase and not with the bound solutes.

Single Isotope Effect. As outlined in the Introduction, isotope effects upon binding constitute a unique probe of molecular interactions because isotopic substitution does not significantly affect potential energy. Potential energy effects cancel between isotopologues, meaning that isotope effects result essentially only from the effects of changes in interactions upon nuclear motions, especially vibrational frequencies.

We have studied perdeuterated solutes as a means of obtaining RPLC separation of isotopologues and accurate values of isotope effects. For purposes of comparison and interpretation, isotope effects for a single deuterium substitution are needed, so we calculate averaged isotope effects per CH/CD bond from our directly observed total isotope effects, TIE, as %IE (eq 4). The

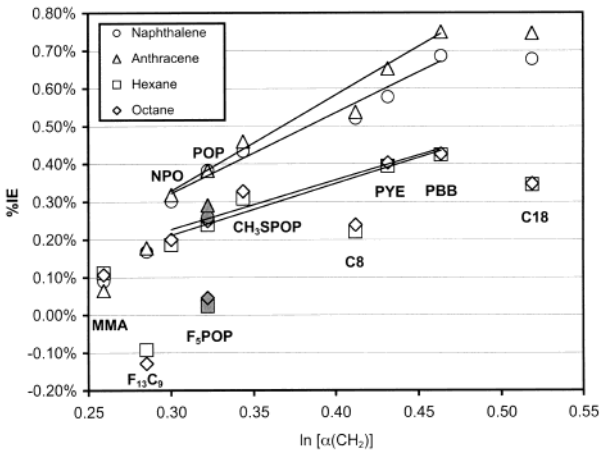

Figure 3. Logarithmic plot (ln−ln) of %IE [equivalent to ln(average isotope effect per H/D) × 100] vs $\alpha(CH_2)$ (retention factor of a methylene unit, eq 2) for four representative solutes and the 10 stationary phases studied, mobile phase 70% methanol/water, 30 °C. Linear regressions are shown for the five aromatic stationary phases (see text). Stationary phases (cf. Figure 1) are MMA = poly(methyl methacrylate), $F_{13}C_9$ = 4,4-di(trifluoromethyl)-5,5,6,6,7,7,7-heptafluoroheptyl, NPO = 3-(*p*-nitrophenoxy)propyl, POP = 3-phenoxypropyl, F_5POP = 3-(pentafluorophenoxy)propyl, CH_3SPOP = 3-(*p*-methylmercaptophenoxy)propyl, C8 = octyl, PYE = 2-(1-pyrenyl)-ethyl, PBB = 3-(pentabromobenzyloxy)propyl, and C18 = octadecyl.

basis of this calculation is the usual approximation that, at ordinary temperatures, the isotope effect for each CH/CD bond contributes additively to the free energy (accurate if the isotope effect is controlled by zero-point energy differences) and thus multiplicatively to the TIE. That is, $\ln(\text{TIE}) = \ln(k_H/k_D) = n \ln(\text{avg single CH/CD IE})$, where *n* is the number of CH/CD substitutions in the deuterated isotopologue, so that the average single CH/CD isotope effect $= (k_H/k_D)^{1/n}$, or, expressing this isotope effect on a percentage basis, $\%\text{IE} = 100[(k_H/k_D)^{1/n} - 1]$.

In any molecule the average %IE cannot be exactly the same except for symmetry-related CH/CD bonds, so most of the molecules we have investigated involve finite differences among different types of bonds. However, since all are CH/CD bonds and are largely nonpolar, the interactions between them and the mobile as well as the stationary phase should be quite similar within a given molecule. We do see differences between aromatic and aliphatic CH/CD bond types (cf. Table 1), but the only molecule studied which contains both aromatic and aliphatic is toluene. For the other molecules at least, the use of averaged %IE should be a good approximation. This conclusion is strongly supported by the results, which show that %IE values are closely clustered for almost all compounds on all 10 stationary phases studied (see Figure 3 above). The fact that only relatively small differences in %IE are seen for different aliphatic chain lengths or for different aromatics demonstrates that there is little effect of structural variation within each series, aliphatic or aromatic. This is especially true for the aliphatic series and can be true only if the %IE for methyl protons and the different methylene protons in different chains are all nearly equal; if any type were significantly different, the average %IE should vary among the different compounds.

The trends of isotope effects on binding relative to the binding affinity of each stationary phase for a single CH_2 group are shown for representative solutes in Figure 3, plotted as %IE (eq 4) vs $\ln[\alpha(CH_2)]$ (eq 2). (Since logarithms are proportional

to free energies, this is equivalent to a free energy vs free energy plot.) The retention factor of a methylene unit $\alpha(CH_2)$ (eq 2) is taken as an approximate measure of the overall affinity or binding ability of each different stationary phase. A linear relationship is not necessarily expected but, as shown in Figure 3, the aromatic stationary phases alone give rather linear plots, suggesting the possibility that interactions with aromatic π electrons may play some role, while the nonaromatic stationary phases appear to form separate groups (see Discussion).

Although isotope effects per CH/CD are small, amounting to fractions of a percent, their values have high reproducibility and thus differences in %IE values are highly significant. High reproducibilities are aided by the fact that isotopologue pairs may be injected simultaneously and separated in a single run, but the reproducibilities are high even in those cases where the isotope effect is small enough to necessitate separate determination of retention times by sequential injection of the protiated and deuterated isotopologues. Total isotope effects, TIE, for solutes containing several deuterium substitutions are typically several percent (cf. Table 1) and are reproducible to ±0.1% in most cases. As a typical example, cyclohexane vs cyclohexane-d_{12} on a C18 stationary phase gives a TIE = 1.0422. A reproducibility of ±0.001 gives, from eq 4, %IE = 0.345 ± 0.008. Thus the differences in %IE observed for the different stationary phases are highly significant (range −0.128 to +0.403, with most differences well outside experimental reproducibility, cf. Table 1).

It should also be emphasized that these seemingly small isotope effects nonetheless lead to complete or nearly complete chromatographic separation of isotopologues in most cases. These separations of course result from the very large numbers of theoretical plates characteristic of the HPLC columns employed. These large numbers of theoretical plates are the feature which allows us to determine small isotope effects with very high reproducibility and accuracy.

Because of the unique nature of isotope effects, these isotope effects specifically measure only changes in the properties of the CH/CD bonds of the solute upon transfer from the aqueous mobile phase to the hydrophobic stationary phase. As a result, the %IE isolates separately the effect of the binding process upon just the solute, i.e., shows how the differences in binding interactions between the two phases affect the properties of the CH/CD bonds of the solute. The fact that the observed values of %IE are different from zero directly shows that the solute has different intermolecular interactions with the hydrophobic stationary phase than with the aqueous mobile phase.

As a primary conclusion, our %IE data show that the solute does not interact with the stationary vs the mobile phase while remaining unperturbed; rather, the properties of the solute are altered, probably via changes in the vibrational frequencies of the CH/CD bonds, as a result of the intermolecular interactions of solute with stationary and/or mobile phases. Positive values of %IE show that the CH/CD bonds are less restricted in the stationary phase than in the mobile phase—usually caused by weakening of the bonds, in this case weaker in the stationary phase relative to the mobile phase. Importantly, such weakening in turn implies that the interactions of the solutes with the relatively nonpolar, hydrophobic stationary phase are stronger than those with the polar, aqueous mobile phase. This result, observed here for most solutes and a variety of stationary phases,

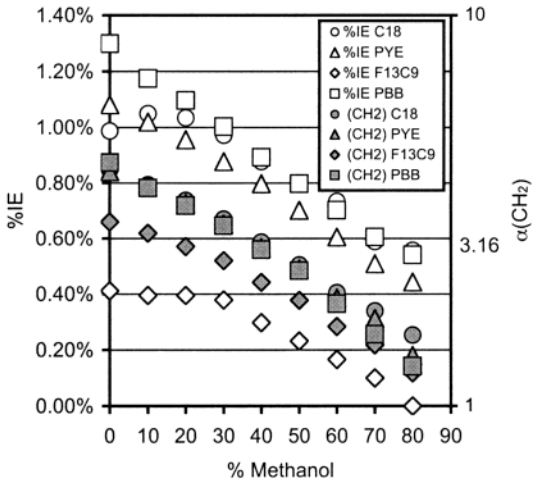

Figure 4. Plot of %IE [equivalent to ln(average isotope effect per H/D) × 100] for benzene (open symbols) and log plot of $\alpha(CH_2)$, the retention factor of a methylene unit (eq 2) (closed symbols) vs percent methanol in the methanol/water mobile phase for representative stationary phases, 30 °C. (Logarithmic plots are presented since logarithms are proportional to free energy differences, so that these plots are equivalent to plots of free energies vs percent methanol.)

is consistent with an active role of the hydrophobic phase in hydrophobic phenomena, i.e., with the idea that lipophilic phenomena make an important contribution to the hydrophobic effect.

The values of %IE are higher for aromatic than for aliphatic CH/CD in all chromatographic systems examined. Moreover, for the aromatic isotopologues %IE increases with the molecular size, while it remains constant for the H/D alkanes. The largest isotope effects are found for the three stationary phases exhibiting the strongest binding as estimated by $\alpha(CH_2)$, for aromatics the order of %IE being PBB > C18 > PYE, for aliphatics, PBB > PYE > C18. In contrast, the smallest isotope effects for aromatic solutes are found for the two stationary phases exhibiting the weakest binding as estimated by $\alpha(CH_2)$, methyl methacrylate ester (MMA) and fluorinated $F_{13}C_9$. For aliphatic solutes, small isotope effects are found for MMA and $F_{13}C_9$, the latter even giving inverse isotope effects for aliphatic solutes, and fluorinated F_5POP gives small isotope effects as well.

These trends in %IE with different types of solutes and stationary phases are also consistent with an active role of the hydrophobic phase in hydrophobic phenomena. They suggest possible involvement of CH/CD---π (aromatic) interactions in the isotope effects and hence in the binding process (see Discussion).

Effect of Mobile Phase Upon Retention and Isotope Effects. While it was not possible to examine most solutes over a wide range of solvent composition, it was possible to determine %IE for benzene over the range of water (0% methanol) up to 80% methanol/water, as shown in Figure 4 along with $\alpha(CH_2)$, the retention factor of a methylene unit, on four representative stationary phases. There are significant changes in %IE which tend to parallel the trends in binding affinities for a methylene unit, with the exception that the %IE for the nonaromatic stationary phases C18 and $F_{13}C_9$ levels off at about 20% methanol and less. Entirely comparable effects are found with all solutes and stationary phases over the accessible range of 60−80% methanol for aromatic solutes and

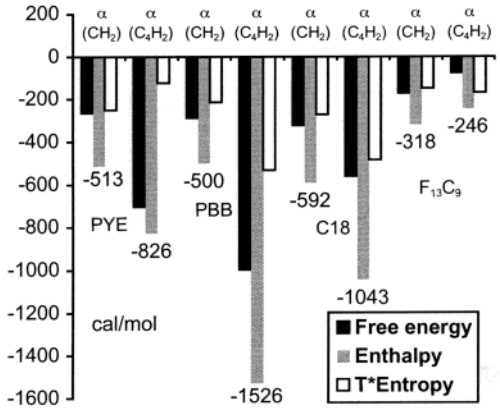

Figure 5. Gibbs free energy ΔG^0, enthalpy ΔH^0, and entropy term $T\Delta S^0$ changes for transfer of aliphatic CH_2 and aromatic C_4H_2 units from 70% methanol/water into four representative hydrophobic phases PYE, PBB, C18, and $F_{13}C_9$, 30 °C. Numerical values of ΔH^0 are shown.

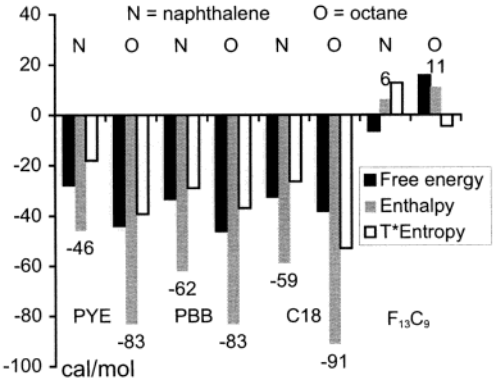

Figure 6. Gibbs free energy ΔG^0, enthalpy ΔH^0, and entropy term $T\Delta S^0$ components of the total isotope effect TIE for transfer of naphthalene and octane from 70% methanol/water into four representative hydrophobic phases, PYE, PBB, C18, and $F_{13}C_9$, 30 °C.

70−80% methanol for aliphatic solutes (complete data for all solutes are in Table A of supporting information).

If the mobile phase simply provided an essentially noninteracting cage for the solute, the isotope effects would be independent of mobile phase composition. The fact that the isotope effects depend on mobile phase composition as well as on stationary phase demonstrates that the solute interacts with the mobile phase, too. Hence, these isotope effects show that the aqueous phase plays an active role as well, in addition to the important contribution to the hydrophobic effect from lipophilic phenomena (see the previous section).

Temperature Effects on Binding and on Isotope Effects. Temperature effects were studied for all H/D isotopologues in 70% methanol/water mobile phase on four representative stationary phases. In all cases, the van't Hoff plots for the measured chromatographic retention factor k were highly linear and show that the binding process is exothermic. As discussed above (see eq 5), k differs from the equilibrium constant for binding K as $K = k/\phi$. The phase ratio ϕ being difficult to determine, thermodynamic quantities involving k are not useful for comparison.

However, ϕ cancels when ratios of k values are taken, meaning that the α factors and isotope effects equal true ratios of equilibrium constants K (cf. eq 5), so that meaningful thermodynamic quantities ΔG^0, ΔH^0, and ΔS^0 can be calculated for the retention factor for a methylene unit $\alpha(CH_2)$, eq 2, the retention factor for an aromatic unit $\alpha(C_4H_2)$, eq 3, and the isotope effect TIE. Figure 5 compares these thermodynamic quantities, taken from van't Hoff plots on four stationary phases, for $\alpha(CH_2)$ and $\alpha(C_4H_2)$; Figure 6, for the TIE of a representative arene, naphthalene, and alkane, octane. The fluoroalkane $F_{13}C_9$ stationary phase is unusual, possibly a reflection of the extremely low dispersive properties of this stationary phase.

The free energy of binding is seen to be dominated by enthalpy rather than entropy in every case. ΔH^0 is uniformly negative, indicating stronger interactions of the solute with the stationary phase than with the mobile phase, and ΔS^0 is uniformly negative as well, indicating decreased freedom of motion of the solute in each stationary phase relative to the aqueous phase. These entropy results would not be expected if the primary source were freeing of solvent molecules organized around the solute in the aqueous phase. Instead, it appears that *solute motional freedom is more restricted in the hydrophobic stationary phases*. This restriction appears not to arise from solute conformational flexibility, since the (C_4H_2) unit is conformationally immobile and is contained within rigid aromatic systems. These results apply to (CH_2) and (C_4H_2) units inserted into, respectively, alkane and aromatic structures, and so they do not necessarily parallel exactly the thermodynamic quantities for complete molecules. But they have the major feature of allowing comparison *among different stationary phases* of the inherent binding effects for structural units within molecules, helping to clarify the origins of the hydrophobic effect.

The isotope effects are dominated by enthalpic contributions in all but one case, that of naphthalene binding to the unusual fluoroalkane $F_{13}C_9$ stationary phase. The latter case could represent an extreme in which interactions with the stationary phase are so weak that the entropic effect primarily reflects an aqueous phase (cavity) effect. Since the isotope effect is determined *only* by interactions of the solute, this result cannot be associated with any freeing of solvent molecules; rather, in this case an entropy-dominated isotope effect must reflect the contribution of changes in low-frequency vibrations or in rotations upon transfer from the aqueous to the hydrophobic phase.

A plausible explanation is that, at least for the flat, rigid molecule naphthalene, solute rotations are inhibited in the aqueous solvent cage but less restricted in the hydrophobic phase. This hypothesis would imply that similar (small) aqueous-phase effects would be present for all other stationary phases, too, but for all except the minimally interacting $F_{13}C_9$ phase, the aqueous-phase effect would be overwhelmed by effects associated with the stationary phase, and the isotope effect would be dominated by solute interactions with the hydrophobic phase. This interpretation is supported by the observation that the isotope effect for the flexible, nonrigid molecule octane is not dominated by entropy contributions, even with the $F_{13}C_9$ phase.

The enthalpy-dominated nature of the remaining isotope effects is just as expected if their origin lies in changes in CH/CD vibrations, especially the high-frequency stretching vibrations, since at the temperatures studied, changes in high-frequency vibrations give isotope effects that are dominated by changes in zero-point energy, an enthalpy term. The negative enthapies, corresponding to isotope effects >1.0, indicate a CH/

CD vibrational frequency decrease in both octane and naphthalene upon transfer from the aqueous phase into the hydrophobic phase. Differences in solvation would be expected to have small effects on vibrational frequencies, with the lower frequency arising from stronger interactions with solvent. In this case, decreased vibrational frequency in the hydrophobic phase implies stronger interactions of hydrophobic phases with CH/CD bonds than in the aqueous phase. Conversely, the isotope effect <1.0 for octane with the very weakly interacting $F_{13}C_9$ indicates that, in this case, interaction of octane with the aqueous phase is stronger than with the $F_{13}C_9$ phase.

Calculation of Differences in Molecular Properties of Isotopologues. The features of the observed isotope effects—in particular, whether they are greater than or less than 1.0, together with the trends found for different stationary phases—give valuable qualitative information about hydrophobic binding, but it would be valuable to obtain further quantitative insights. As discussed above, vibrational frequency changes appear to be the major source of the isotope effects we have observed. Hence, an investigation of solute vibrational frequencies might provide further support and interpretation of our results. However, changes of only a few cm^{-1} in the stretching frequency of each CH/CD bond would be sufficient to account for the magnitude of the observed isotope effects.

It should be emphasized that changes in solvation would not be expected to alter the vibrational frequencies of the nearly nonpolar CH/CD bonds a great deal, so that even such small effects can yield insights into differences in solvation in the aqueous vs the stationary phase. Moreover, isotope effects constitute a very sensitive probe, so that effects of the magnitude reported here are significant and readily interpretable. And importantly, as discussed above, our isotope effects have very high reproducibilities and hence can justifiably be used to probe phenomena such as the nature of, and differences in, hydrophobic binding phenomena.

Though frequency changes of only a few cm^{-1} are expected, we did undertake preliminary FT-IR studies of certain of our solutes in different solvents. Small shifts of approximately the appropriate magnitude to account for the observed isotope effects were found in some but not all cases. Consequently, and not surprisingly, interpretation was not feasible without more data, being complicated by the fact that these solution spectra result from superposition of several normal vibrations involving multiple CH or CD bonds present in each solute as well as by the smallness of the frequency shifts expected (and observed).

It is feasible, however, to explain our results further through computations of molecular properties. Such a theoretical rationale is valuable both in supporting the interpretation of the present results and as a potential tool for future extrapolation and prediction.

The chromatographic retention mechanism can be defined as the sum of all physical interaction processes among solute, stationary phase, and mobile phase.[11] Among all the H/D pairs of compounds examined, only nitrobenzene possesses a large dipole moment. For the remaining compounds, the major interactions to be considered are dipole−induced dipole and London dispersion interactions between the solute and the stationary phase and additionally the hydrophobic (solvent) cavity effect. The van der Waals volume (V^{vdW}) and surface area (S^{vdW}) are commonly used for describing interactions of nonpolar molecules. Also, the volume-related molecular polarizability (α) describes satisfactorily the dispersive properties of nonpolar solutes[38] and is also related to the hydrophobic cavity effect.

van der Waals Radius, Surface Area, and Volume. Experimental values for carbon, oxygen, and nitrogen van der Waals (vdW) atomic radii (r) were used to calculate V^{vdW} and S^{vdW}.[39] For hydrogen, however, we introduced corrections to adjust vdW radii for sp^2 vs sp^3 carbon-bound hydrogen.[40] These corrections are detailed in the supporting information.

We then calculated the change in the vdW radius for the deuterium atom. According to Pauling's model, the vdW radius is described by eq 8, where b is a number characteristic for an element.

$$r = \text{const} + b \tag{8}$$

As described in the Introduction, the amplitude of vibrations is different for H and D. The smaller zero-point energy of vibrations (ZPE) of deuterium bound to a carbon atom is a manifestation of the different vibrational frequencies of deuterium and protium in the lowest vibrational state. Pictorially, one may think of deuterium as "penetrating" a smaller area of the potential energy surface.

The turning point, X_{tp}, of a quantum harmonic oscillator in the lowest vibrational state is given by eq 9, where μ_{CH} is the reduced mass of H (bound to C), ω is the CH bond vibrational frequency (in rad s^{-1}),

$$X_{tp} = (\hbar/\mu_{CH}\omega)^{1/2} \tag{9}$$

[$\omega = (\kappa/\mu_{CH})^{1/2}$, where κ is the force constant], and $\hbar = h/2\pi$, where h is Planck's constant. X_{tp}, in the classical limit, corresponds to the amplitude of a harmonic oscillator. Following Ubbelohde,[41] the extent, or amplitude, of vibration is assumed to be correlated with the vdW radius. Therefore, b (eq 8) is simply postulated to be equal to X_{tp}. The frequency of the CH vibration was estimated from experimentally measured IR spectra of protiated solutes.[42] For an sp^3 CH bond the average frequency is 2934 cm^{-1}, for sp^2 (aromatic) CH, 3067 cm^{-1}. Using eq 9 we obtained values for b of 0.103 and 0.101 Å, respectively. The different vdW radii between hydrogen (and deuterium) bound to an sp^2 carbon (smaller) and an sp^3 carbon (larger) give different H/D volumes, which contribute to differences in hydrophobicity of aromatic and aliphatic compounds, since hydrophobic properties are related to molecular volume.

The difference in vdW radii between protium and deuterium results from the difference in amplitude of vibrations of CH vs CD bonds. Since b is proportional to $1/(\mu\kappa)^{1/4}$, the ratio of b_H for protium to b_D for deuterium is given by eq 10, from which

(38) In general, such a picture is certainly too simplistic. However, since we consider only two families of solute molecules, namely, the aromatic and aliphatic hydrocarbons that share general patterns and shapes of electronic density, the assumption that polarizability is proportional to volume within each family should work quite well.
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eq 11 and (using eq 8) eq 12 follow.

$$b_H/b_D = (\mu_{CD}/\mu_{CH})^{1/4} = (13/7)^{1/4} = 1.1674 \quad (10)$$

$$b_D = b_H - 0.1434 b_H \quad (11)$$

$$r_D = r_H - 0.1434 b_H \quad (12)$$

The modified values of vdW radii for deuterium atoms bound to sp^2 and sp^3 carbon were then included in calculations of the vdW surface areas and volumes of the isotopologues we have studied (see Tables D and E in the supporting information). The differences between H and D compounds are given as $\Delta S^{vdW} = S_H{}^{vdW} - S_D{}^{vdW}$ and $\Delta V^{vdW} = V_H{}^{vdW} - V_D{}^{vdW}$.

Polarizability Change upon Deuteration. Isotope effects of H/D substitution on molecular polarizability, α, and refractive index have been related to the changes in the zero point energy (ZPE) of vibrations, and the polarizability isotope effect (PIE) was correlated with vibrationally averaged transition dipole moments using an argument based upon perturbation theory.[43] In our work we used a simplified approach to estimate PIE assuming additivity of atomic contributions to molecular polarizability. Atomic polarizabilities, in turn, are related to the vdW radius according to the Slater−Kirkwood approximation.[39] Details of the Hamiltonian employed may be found in the supporting information, along with the values of atomic and molecular polarizabilities of the isotopologues (Tables D and E). Again, the difference in vdW radii between isotopologues results in different atomic and, consequently, molecular polarizabilities between H and D compounds, expressed as $\Delta\alpha = \alpha_H - \alpha_D$.

Being small, the changes in molecular properties brought about by isotopic substitution are not easily measured experimentally. Therefore, we will employ these theoretical estimates of isotopic differences to correlate and explain our experimental data on binding and isotope effects.

QSRR Analysis of the Total Isotope Effect. To investigate more quantitatively the nature of the differences in intermolecular interactions of the stationary and mobile phases with solute molecules, we performed QSRR analysis of the experimental isotope effects. In this case, too, it should be emphasized that these isotope effects specifically measure only changes in the properties of the CH/CD bonds of the solute upon transfer from the aqueous mobile phase to the hydrophobic stationary phase and thus isolate separately the effect of the binding process upon just the solute.

Molecular size descriptors in QSRR have been employed to elucidate the mechanisms of chromatographic binding.[44] Figure 7 represents the trends of the total isotope effect, TIE, with a 70% methanol/water mobile phase vs the best descriptor found—the difference in van der Waals volumes between isotopologues, ΔV^{vdW}, for each solute. With all stationary phases, there is a clear distinction between aliphatic and aromatic isotopologues. For most of the compounds, there are clear linear trends within the aliphatic and aromatic groups separately, so additional correlation analysis was done separately for each group. The correlation coefficients, along with the equation significance

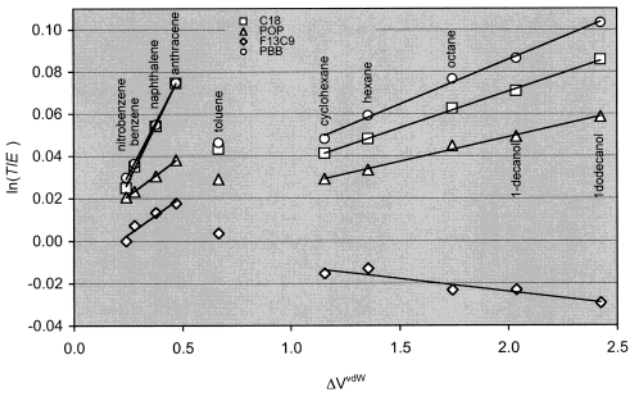

Figure 7. Total isotope effect TIE for transfer from 70% methanol/water into stationary phases vs ΔV^{VdW}, 30 °C. Linear regressions for aromatic and aliphatic solutes are shown. Toluene, having both aromatic and aliphatic CH/CD bonds, shows intermediate behavior and is omitted from the regressions.

levels, are presented in Table F of the supporting information. QSRR analysis was done for the group of all isotopologues, as well as for the five separate alkanes, all five arenes, four arenes (without toluene, since toluene contains both aromatic and aliphatic CH), and three arenes (without toluene and nitrobenzene, since nitrobenzene is special in being so highly polar). These results imply that ΔV^{vdW} may serve as a useful, empirical chemometric predictor of isotope effects in hydrophobic binding and in reversed-phase HPLC separations.

Discussion

The goal of this investigation was to elucidate further the factors that contribute to the phenomenon of hydrophobic binding. A better understanding of the interactions involved would elucidate both the factors affecting separation in reversed-phase HPLC and the nature of hydrophobic interactions in organic and biological chemistry.

Our QSRR analysis of binding to the 10 reversed-phase HPLC stationary phases we investigated (cf. Results) showed that the main driving force for binding is strongly dependent on the solute's McGowan characteristic volume. As indicated in Results, our QSRR data indicate that the binding to all of our stationary phases, even including the *p*-nitrophenyl-containing phase NPO, is primarily hydrophobic in nature.

The hydrophobic binding phenomenon is complicated by the fact that the free energy of binding includes a combination of effects—both on the solute itself and on the aqueous and hydrophobic phases—making it very difficult to determine the kinds of interactions that may be involved. By examining isotope effects upon binding of deuterated vs protiated solutes, we were able to dissect the binding process and probe just the changes in interactions of the solute in the aqueous (mobile) vs the hydrophobic (stationary) phases. This ability results from the fact that isotopic substitution does not significantly affect potential energy, so that potential energy effects cancel between isotopologues and, therefore, isotope effects result essentially only from the effects of changes in interactions upon nuclear motions, especially vibrational frequencies. As shown in Results above, our measured values have high reproducibility and thus differences between different types of solutes and stationary phases are highly significant.

For these reasons, our observed %IE values isolate the effect of binding upon the solute alone and thus show directly any

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differences in binding interactions of the solute in the hydrophobic, stationary phase vs the aqueous, mobile phase. The fact that the observed values of %IE are different from zero conclusively shows that the interactions of the solutes with the hydrophobic phase are different from their interactions with the aqueous phase. This already constitutes the first important finding demonstrated by our experiments: *the solute is perturbed upon transfer from the aqueous phase to the hydrophobic phase; i.e., hydrophobic binding is not simply a matter of how the aqueous vs the hydrophobic phase solvates the solute, it also specifically involves different interactions of the solute with the aqueous vs the hydrophobic phase, which affect the properties of the solute as well.*

Positive values of %IE (eq 4) arise when the protiated solute binds to the stationary phase more strongly than the deuterated solute. This requires that the CH/CD bonds of the solute are less restricted in the stationary phase than in the mobile phase. Less restricted motion indicates lower force constants in the stationary phase than in the aqueous phase, i.e., weakening of the CH/CD bonds in the hydrophobic phase relative to the aqueous phase. This in turn indicates that the interactions of the solutes with the relatively nonpolar, hydrophobic stationary phases are *stronger* than those with the polar, aqueous mobile phase. Our experiments show that this weakening occurs for most solutes with a variety of stationary phases. This leads to the second important point demonstrated by our experiments: *the hydrophobic phase appears to play an active role in hydrophobic phenomena; i.e., lipophilic phenomena make an important contribution to the hydrophobic effect.*

As shown above in Results, values of %IE differ for different types of solutes and stationary phases, also indicating an active role of the hydrophobic phase in hydrophobic phenomena. In fact, the results suggest the possible involvement of CH/CD---π (aromatic) interactions as contributing to the observed differences in isotope effects and hence in the binding process among different types of solutes and hydrophobic phases. Figure 3 shows representative data, but the same trends are found in all of our data, involving 10 different solutes (cf. Table 1) and different mobile phase compositions (cf. supporting information, Table A). A monotonic, rather linear increase in %IE with $\ln[\alpha(CH_2)]$, where $\alpha(CH_2)$ is the binding strength for a CH_2 unit, is found for the five aromatic stationary phases studied. Even the aliphatic stationary phases C18 and C8 give lower isotope effects than would be expected based on their binding strength and their comparison with the isotope effects seen on the aromatic stationary phases. Moreover, aromatic solutes (that is, aromatic CH/CD bonds) all exhibit higher %IE than aliphatic solutes (that is, aliphatic CH/CD bonds) for all stationary phases except the very weakly binding, very polar, MMA phase.

Our temperature-dependence studies also show that the binding process is enthalpy-driven (cf. Figure 5). QSRR analysis also indicates that the McGowan characteristic volume of the solute is the major variable enhancing solute binding to all of these stationary phases (cf. eq 6 and Table 2). In addition, our calculations of ΔV^{VdW}, the difference in van der Waals volumes between isotopologues, reveal that it is the best descriptor of the changes in %IE among different stationary phases (cf. Figure 7). The fluorous stationary phase $F_{13}C_9$ shows an inverse dependence of %IE on ΔV^{VdW}, entirely consistent with the fact that such fluorous phases, including fluorous solvents, interact very weakly with, and phase-separate from, both polar and hydrocarbon phases.[45] Taken together, the data mutually support the conclusion that binding to the stationary phases we have studied is dominated by London dispersion forces between the solute and the stationary phase.

The differences noted between %IE for aromatic vs aliphatic stationary phases and solutes lead to a plausible model which qualitatively explains these trends, as follows. It is well-known that aromatic rings have favorable edge-to-face interactions, including the herringbone structure for crystalline benzene.[46] It is also known that aliphatic CH complexes favorably with the π-face of aromatic rings.[47] Such interactions appear likely to be present in the binding of solutes and stationary phases when either or both contain an aromatic group. It would not be necessary for solutes to be bound entirely through such complexation, but if on the average there were significant contributions from binding involving aromatic π faces, this should directly affect the isotope effects. In particular, analogously to hydrogen bonding, but a much smaller effect, the binding of CH/CD to an aromatic π face should lower the CH/CD stretching vibrational frequency, and we suggest it is probable that this small frequency lowering effect would still be larger than the frequency lowering effect that would be produced by generalized London dispersion interactions. But also, we believe that use of aromatic π electrons to form such complexes with CH bonds would, by this small degree of electron-withdrawal from the ring carbons, lower by a small amount the stretching frequency of the CH/CD bonds of an aromatic solute, which should then give enhanced isotope effects.

According to this model, the relatively high %IE values for aromatic solutes binding to aromatic stationary phases would be the result of relatively strong aromatic CH/CD edge interactions with the aromatic stationary phases. The lower, but still relatively high, isotope effects seen for aliphatic solutes binding to aromatic stationary phases would be the result of effective aliphatic CH/CD interactions with the aromatic stationary phases, which would give enhanced isotope effects, though not as large as those resulting from aromatic−aromatic edge interactions. The isotope effects for aliphatic solutes binding to aliphatic stationary phases C18 and C8 would not involve any of these aromatic interactions and so would be smaller, but binding of aromatic solutes to the aliphatic stationary phases would involve interaction of the π face of the aromatic solute with the aliphatic groups of the stationary phase, giving enhanced isotope effects for aromatic as compared with aliphatic solutes binding to aliphatic stationary phases.

We have also investigated plots of %IE vs $\ln[\alpha(C_4H_2)]$, where $\alpha(C_4H_2)$ is the binding strength for an aromatic C_4H_2 unit, but the correlation of isotope effect with binding strength of the aromatic unit is not as good as with $\alpha(CH_2)$, even for aromatic solutes with aromatic stationary phases. We believe this results from the more specific interactions of aromatic groups that our model suggests. In this sense, the values of α-

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(CH_2) may be thought of as reflecting less specific interactions involving London dispersion forces along with, in the case of the aromatic stationary phases, the characteristic attractive interaction between an aliphatic CH_2 group and the π clouds of the aromatic stationary phases. Thus, use of $\alpha(CH_2)$, as in Figure 3, led to more readily interpretable differences and suggested the model we are proposing here.

The fluorous stationary phases $F_{13}C_9$ and F_5POP would be expected to have very weak interactions with hydrocarbon solutes.[45] The enthalpy measured for binding of a CH_2 unit to the $F_{13}C_9$ stationary phase is indeed considerably less negative than that for other stationary phases (cf. Figure 6). It would then be possible that aliphatic solutes would have stronger London dispersion interactions with the methanol present in the mobile phases and that, at sufficiently high percent methanol, the total interactions with CH/CD bonds of the solute could be larger within the mobile phase than within the $F_{13}C_9$ phase. As a result, the CH/CD bonds would have slightly lower stretching vibrational frequencies in the mobile phase than in the fluorous phase, which would in turn result in inverse isotope effects (%IE <1), as observed for all five aliphatic solutes we have studied (70% methanol−water mobile phase, cf. Table 1). Inverse isotope effects are also found for all five aliphatic solutes binding to $F_{13}C_9$ for mobile phase compositions of 80% methanol−water and 60% acetonitrile−water (cf. supporting information, Table A).

However, aromatic solutes give normal (%IE greater than 1), though quite small, isotope effects upon binding to the $F_{13}C_9$ stationary phase. Both the binding of an aromatic C_4H_2 unit and its enthalpy of binding are significantly weaker than for the aliphatic CH_2 unit. A possible interpretation of this weak binding for the aromatic C_4H_2 unit is that, upon binding, aliphatic solutes do to some extent penetrate into the fluorous $F_{13}C_9$ phase, but aromatic solutes may not penetrate so well in view of possible repulsive interactions between the polar C−F bonds and the π electron clouds of the aromatic solutes. On the other hand, the weak interactions of aromatic solutes might well involve weak attractive interactions of the polar highly electronegative fluorine atoms with the aromatic H/D atoms, i.e., weak CH/CD---F hydrogen bonding, which should cause lower CH/CD stretching frequencies and thus give normal isotope effects. These effects are small, and so this can only be a suggestion at this point.

Finally, the MMA (methyl methacrylate ester) stationary phase exhibits even weaker binding of a CH_2 unit than the $F_{13}C_9$ phase. The unique feature of MMA is that the isotope effects are very similar for binding of both aliphatic and aromatic solutes. This similarity may result from the absence of specific interactions with aromatic solutes and in that sense supports our model, which explains differences in terms of characteristic π face and/or edge interactions. However, of all the stationary phases studied, MMA is probably most likely to have significant amounts of adsorbed methanol equilibrated from the mobile phase, and this methanol could also be involved in the binding process. From the very weak binding observed, as well as the suggested interpretation of the similarity of isotope effects for aromatic and aliphatic solutes, it may be that such methanol effects are minimal. But, because the possible complications of this system cannot be ignored, any conclusion about MMA must remain speculative.

It might be suggested that aromatic solutes, being more polarizable than aliphatic, could have some interaction with the silica core of the stationary phases to account for the larger isotope effects seen in the aromatic series. However, we consider this possibility to be very improbable based on our QSRR results discussed above, which indicate a binding mechanism dominated by London dispersion forces. In addition, the fact that larger isotope effects are observed for larger aromatic solutes strongly argues against this interpretation, since the larger aromatics should have less access to the inner core. At the least, fewer of a larger aromatic solute's CH/CD bonds could interact simultaneously with the core, so that the larger the aromatic solute the less the average isotope effect per CH/CD should be affected by the core, contrary to what is observed.

The model presented here constitutes our third point, suggested by our experiments: *the active role of the hydrophobic (stationary) phase can be explained by a plausible model which invokes specific contributions—from (a) aromatic edge-to-face attractive interactions and (b) attractive interactions of aliphatic groups with the π clouds of aromatic groups present as the solute or in the stationary phase.*

So far we have discussed our studies of different types of stationary phases, which have shown that the hydrophobic phase plays an active, attractive role in binding. Binding equilibria are of course controlled by the difference in free energies of the solute in the stationary phase vs the mobile phase. To elucidate possible interactions of solutes within the mobile phase, further investigation of the effect of the mobile phase composition on the isotope effects was undertaken (cf. Figure 4). It is observed, as qualitatively expected, that isotope effects decrease at higher percent methanol in the mobile phase, in fact showing a strong parallel with changes in $\alpha(CH_2)$, the binding strength for a CH_2 unit over the same range of methanol concentrations. A leveling of isotope effects between 30%−20% and 0% methanol is observed for only the C18 and fluorous stationary phases, but not for PYE or PBB (aromatic) phases. This leveling effect is difficult to understand at present, at least without trying to invoke highly specific and speculative interactions. The problem is that, since it involves a different type of dependence on mobile phase composition for different stationary phases, it cannot result directly from any interactions within the mobile phase, since the solute and its interactions with the different mobile phase compositions are the same no matter which stationary phase is being studied. Hence, the source of this leveling effect must reflect phenomena associated with the stationary phases that would be somehow affected by methanol concentration in different ways for the different types of stationary phases. Further experiments on a variety of solutes seem necessary to test the generality and nature of this leveling effect.

Aside from these leveling effects, however, the changes in isotope effect with mobile phase composition can be explained by increased attractive interactions with the solute by mobile phases of increasing methanol concentration. Such increases would be expected if the nonpolar methyl groups of methanol molecules could orient toward and possibly complex with the solute molecule, resulting in a more hydrophobic solute environment within the mobile phase, with interactions that start to resemble those of the solute within the stationary phase. This in turn would decrease any differences in the interactions of

CH/CD within the stationary phase vs the mobile phase and so decrease the isotope effect. In fact, a possible explanation of these rather significant changes in isotope effects would be along the lines of our model above. If methanol forms complexes with the π faces of aromatic solutes, the change in isotope effects with increasing mobile phase methanol concentrations might be enhanced and might be a larger effect than for aliphatic solutes, which are expected to have less specific London dispersion type interactions with methanol. This interpretation was suggested by experimental and theoretical evidence supporting complexation of ethanol with aromatic rings as the source of observed antihydrophobic cosolvent effects on rates of several organic reactions.[48] Although a range of solutes has not been studied over a wide range of methanol compositions, we have determined isotope effects for all 10 solutes investigated with 80%, 70%, and in some cases 60% methanol (cf. supporting information, Table A). With very few exceptions, mostly involving the polar nitrophenyl NPO and fluorous $F_{13}C_9$ stationary phases, plots of %IE vs percent methanol uniformly give slopes about half as great for aliphatic as for aromatic solutes. Hence the fourth point derived from our experiments is that *the sensitivity of isotope effects to changes in mobile phase composition is approximately half as great for aliphatic as for aromatic solutes, results which are consistent with more specific interaction of aromatic solutes with methanol, probably involving HOCH$_3$---π face complexation.*

Though our data on temperature dependence are limited to four representative hydrophobic (stationary) phases and only a few solutes (cf. Figures 5 and 6), they indicate that the transfer from aqueous to hydrophobic phase is dominated by a negative enthalpy contribution, meaning that solutes are more strongly bound by their interactions with the hydrophobic phase than those with the aqueous phase. The entropy change is also found to be uniformly negative, showing that the motional freedom of the solute is actually more restricted in the hydrophobic phase than in the aqueous phase. These results lead to our fifth point: they imply that *the hydrophobic phase packs and folds around the solute to give significant binding interactions that appear also to restrict the motional freedom of the solute, while any aqueous solvent cage around the hydrophobic solutes interacts less strongly and gives less restriction of motional freedom.*

Our calculations of isotopic property differences for different solutes show that the difference in van der Waals volume of isotopologues, ΔV^{vdW}, for each solute is the best descriptor found for correlating the observed isotope effects (cf. Figure 7). This empirical correlation provides a potentially useful means of evaluating the isotope effects to be expected for more complex structures. We should emphasize that ΔV^{vdW} is a specific (calculated) property of individual solute molecules, a property which we find *empirically* to be a good predictor of isotope effects. The fact that ΔV^{vdW} is a good predictor, although it involves volume, not surface area (ΔS^{vdW}, the corresponding surface area difference, was found to be a less effective predictor of the observed isotope effects), suggests that ΔV^{vdW} provides a combined measure of possibilities for interactions and in some empirically appropriate way approximately combines aspects of surface area plus aspects of polarizability (expected to be volume-dependent). This specific molecular property reflects the nature of all of the molecule's CH/CD bonds taken together, but does not depend directly on other parts of the molecular structure, and thus in turn reflects the capacity of the molecule for interactions that would give altered CH/CD vibrational frequencies upon transfer from the aqueous to the hydrophobic phase, resulting in isotope effects. Thus our sixth point is that *ΔV^{vdW} may serve as an empirical, calculable chemometric predictor of isotope effects for diverse structures.*

In addition, these calculations serve as a sort of corroboration of our interpretations of the observed isotope effects; that is, although we cannot directly make use of infrared data since the CH/CD vibrational frequency shifts on transfer from the aqueous to the hydrophobic phase are small, these computations provide an equivalent, alternative measure of expected isotope effects which is both consistent with observations *and* capable of being used for predictive purposes for other structures.

Our data demonstrate significant molecular interactions with the solute upon its binding into hydrophobic environments. The data also suggest interactions of methanol with the solute when it is in the aqueous water−methanol environment. A model which invokes relatively specific aromatic edge-to-face and alkyl−π face interactions with aromatic solutes as well as aromatic groups present as part of hydrophobic phases is found to explain the differences observed among aromatic and aliphatic structural types. While less specific interactions could conceivably explain our observations, the major trends observed, all of which are consistently explained by our model, do rather strongly support this model at least as a working hypothesis.

Conclusions

We have investigated the effects of (a) solute structure including both aliphatic and aromatic solutes, (b) hydrophobic (stationary) phase structure, and (c) hydrophilic (mobile) phase composition on the equilibrium transfer of solutes from hydrophilic to hydrophobic phases. In this way, we are able to determine the importance and investigate the nature of each of these three variables. In particular, by study of solute isotopologues we determined isotope effects, which constitute a unique probe of hydrophobic binding, i.e., of the transfer of the solute from the aqueous to the various hydrophobic phases. From this information we draw the following conclusions.

(1) The fact that the isotope effects change significantly with different hydrophobic (stationary) phases demonstrates that lipophilic phenomena make an important contribution to the hydrophobic effect. The solute is not passively transferred without change from the hydrophilic to the hydrophobic environment, but rather the solute itself is perturbed by the change from one environment to the other.

(2) The fact that isotope effects are uniformly >1 (with the exception of one fluorous stationary phase, $F_{13}C_9$, which is unique in having simultaneously hydrophobic and lipophobic properties)[45] shows that solute CH/CD bonds have *stronger* interactions in hydrophobic environments than in hydrophilic environments.

(3) The differences in isotope effects among aromatic vs aliphatic solutes and among aromatic vs aliphatic stationary phases can be nicely explained by a plausible model which invokes relatively specific aromatic edge-to-face and alkyl−π face interactions with aromatic solutes as well as aromatic groups present as part of hydrophobic phases.

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(4) We find that mobile phase interactions, at least in the case of methanol−water mobile phases, also influence the isotope effects; that is, the isotope effects are partially determined by interactions of at least the mobile phase component methanol with solutes and are not solely dependent on interactions of solutes with the hydrophobic phase. The fact that the sensitivity of isotope effects to aqueous (mobile) phase composition (percent methanol in methanol−water mobile phases) is approximately half as great for aliphatic as for aromatic solutes is also consistent with this model, in that specific interactions of aromatic solutes with methanol in the mobile phase, probably involving $HOCH_3$---π face complexation, would be expected to cause an increased sensitivity to solvent composition of the isotope effects for aromatic solutes.

(5) The enhanced binding of solutes within the hydrophobic phases also gives entropy effects which indicate that the hydrophobic phases restrict the motional freedom of solutes even more than any possible solvent cages present around hydrophobic solutes in the aqueous phase.

(6) The finding that ΔV^{vdW} is the best empirical molecular property descriptor for chemometric correlation of our observed isotope effects implies that calculated values of ΔV^{vdW} may serve as a valuable predictor of isotope effects for diverse structures in hydrophobic binding and in reversed-phase HPLC separations.

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Supporting Information Available: Tables A−F, containing details of adjustments to van der Waals radii and calculation of atomic and molecular polarizabilities for isotopologues. This material is available free of charge via the Internet at http://pubs.acs.org.

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