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SEPARATION OF MACROMOLECULES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

PORE-SIZE AND SURFACE-AREA EFFECTS FOR POLYSTYRENE SAMPLES OF VARYING MOLECULAR WEIGHT

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SUMMARY

The separation of polystyrenes of varying molecular weight by reversed-phase high-performance liquid chromatography has been studied, using both isocratic and gradient elution with tetrahydrofuran–water mobile phases. Bonded-phase Zorbax® and Zipax® particles (C_{18}-silica) of different pore-diameters were used: 6, 15, 30 and 100 nm. The results confirm that the normal exclusion of high-molecular-weight random-coil polymers from small pores (*e.g.*, 6 nm) has little effect on the reversed-phase retention process. That is, effectively all of the bonded phase within the particle is available for interaction with these sample molecules. Quantitative relationships are derived which permit the interpretation of the present data and its extrapolation for the optimization of reversed-phase separations of polystyrene mixtures.

INTRODUCTION

The separation of macromolecular samples by high-performance liquid chromatography (HPLC) is almost 20 years old. Early applications of size-exclusion chromatography allowed the determination of molecular weight distributions of synthetic polymers (*e.g.*, ref. 1). Within the past decade it was found possible to replace the classical ion-exchangers for protein separations with analogous HPLC packings, resulting in a major advance in separation power and speed (*e.g.*, ref. 2). More recently, considerable interest has developed in the use of reversed-phase separations for samples such as peptides and proteins (*e.g.*, refs. 3–8). From a practical standpoint, the latter technique may eventually prove the method of choice for separating mixtures of macromolecules. Reversed-phase packings are among the most stable and reproduc-

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ible, the columns generally yield state-of-the-art efficiencies, and a wide choice of mobile phase compositions are available for controlling the separation of a given sample.

While such reversed-phase separations of proteins and related samples have demonstrated a considerable practical potential, and underlying understanding of the basis of these separations has yet to be achieved. Because of the large molecular dimensions of the compounds of interest, it is known that pore-size effects are important in the resulting separations, but no general description of the effect of particle pore size on either retention or column efficiency has yet been advanced. A good understanding of the basis of separation of small molecules by reversed-phase HPLC is well underway (*e.g.*, refs. 9–13), even if major questions concerning the retention mechanism are still being argued.

The reversed-phase separation of macromolecules differs in another respect from the corresponding separation of smaller compounds. Whereas the latter separations are normally carried out under isocratic conditions, the separation of proteins and other large molecules generally requires gradient conditions. Moreover, the conditions for optimized gradient separation of macromolecules by reversed-phase HPLC are predicted to be substantially different from those for compounds in the molecular weight range 200–1000 (ref. 14), and these differences have recently been confirmed experimentally[15]. Further study of this aspect of macromolecular separations by reversed-phase HPLC is clearly warranted.

The present study describes a beginning at better understanding the reversed-phase separation of macromolecules. Because the separation of biomolecules such as proteins is complicated by many effects peculiar to this class of compounds, we have begun with a simpler model system: the chromatography of polystyrenes in mixtures of tetrahydrofuran–water. The latter system affords ample opportunity to explore the basic contribution of molecular size to separations by reversed-phase, with none of the added complications characteristic of proteins: secondary adsorption onto the polar silica matrix, irreversible changes in configuration of the protein molecule as a function of separation conditions, shear degradation, etc. Hence we will describe the dependence of polystyrene retention on molecular size and separation conditions, and we will interpret these data in terms of a simple model of the retention process. We will further compare these separations in the gradient *vs.* isocratic elution modes, and relate such separations to present theory for small-molecule samples.

EXPERIMENTAL

Apparatus

The liquid chromatograph used was a DuPont Model 8800 with a Model 850 fixed-wavelength (254 nm) photometric detector (DuPont Instruments, Wilmington, DE, U.S.A.).

Reagents

HPLC-grade tetrahydrofuran (THF) (Fisher Scientific, Pittsburg, PA, U.S.A.) was used with purified water from a Milli-Q system with Organix-Q cartridge (Millipore, Bedford, MA, U.S.A.) for the mobile phases described here. Polystyrene standards (molecular weights of 800, 2000, 4000, 9000, 17,500, 50,000, 100,000, 233,000)

were obtained from Pressure Chemical (Pittsburgh, PA, U.S.A.). These were nominally mono-disperse samples (M_w/M_n values of 1.05–1.08); partial separation into oligomers was evident for samples with molecular weights less than 9000.

Columns

Four different column packings were studied, each of which is based on a silica particle with a monolayer coverage of dimethyloctadecylsilyl (C_{18}) groups (maximum surface coverage with end-capping by trimethylsilyl groups). Zorbax®-ODS is a commercially available product (DuPont) with a particle size of 6 μm and an average pore diameter of 6 nm. Two similar products were prepared by us from Zorbax-SIL® having pore diameters of 15 and 30 nm, respectively. The fourth column packing is a pellicular material based on Zipax® (DuPont), having a particle diameter of 30 μm and a pore diameter of 100 nm. All packings were used in 25 × 0.46 cm columns, packed with a slurry-packing procedure. See Table I for further details.

Procedure

Retention data were measured for both isocratic and gradient elution of the polystyrene standards from the various columns described above. Samples were injected as solutions in THF (3 mg/ml) in most cases, using either 10- or 50-μl sample-loops. It was found that injection of lower-molecular-weight samples dissolved either in pure THF or in an isocratic mobile phase gave identical retention data, and retention times were constant as the mass of injected sample (polystyrene) was varied from 0.03 to 3.2 mg. Unless otherwise noted, retention data were measured at ambient temperature (23 ± 1°C), with a flow-rate of 2.0 ml/min.

The column dead-time, t_0, was taken as the retention time for toluene as sample and THF as mobile phase. For the Zorbax-ODS column, t_0 as measured for toluene (1.24 min) was slightly larger than for uracil as sample (1.12 min). These small differences in t_0 did not significantly affect final calculated values of capacity factor, k', and the value of t_0 for toluene was assumed to be correct.

The reproducibility of retention times was determined for both the same column and for different columns of the same type. In most cases, replicate retention times agreed within 1–2%. In one case, two different 30-nm-pore columns gave t_0 values that differed by 6%, leading to proportionate changes in retention times. The

TABLE I

CHARACTERISTICS OF VARIOUS C_{18}-SILICA PACKINGS USED IN PRESENT STUDY

Surface area and pore diameter values from nitrogen adsorption onto unbonded silica; weight packing in column is for bonded phase.

Particle	*Surface area (m^2/g)*	*Average pore diameter (nm)*	*Weight packing in column (g)*	*Surface area per column (m^2)*
Zipax (30 μm)	0.95	100	6.15	5.8
Zorbax (6 μm)	45	30	3.10	139
	128	15	3.26	417
	304	6	3.29	1000

precision and reproducibility of retention time measurements for isocratic elution of the higher-molecular-weight polystyrene was poorer than the above data for oligomers from the 800-mol.wt. polystyrene. For example, the 50,000-mol.wt. polystyrene showed a coefficient of variation, C.V., for replicate measurements of retention time equal to 6%.

Retention times for individual oligomers from lower-molecular-weight polystyrenes were determined in the usual way —either manually or by means of a data processor (SP4100; Spectra Physics, Santa Clara, CA, U.S.A.). For the higher-molecular-weight polystyrenes, the conditions of separation sometimes resulted in partial resolution of oligomers and the formation of an asymmetrical band, so that the band maximum no longer corresponded to the mean molecular weight. In these cases, the retention time was determined for the midpoint of the band on an area basis.

All gradient elution runs were carried out with linear gradients. Gradients typically began at 20, 40 or 60% (v/v) THF–water and ran to 100% (v/v) THF. Interpretation of retention data from gradient elution studies required that the lag-time, t_L, of the HPLC system be known; t_L is the time required for mobile phase to flow from the gradient mixer to the column inlet. The lagtime was determined by running a gradient with a UV-absorbing solvent as one of the two mobile phase solvents. For the presently used HPLC system, t_L was found equal to 2.2 min.

THEORY

Isocratic retention

The retention of large molecules such as polystyrene oligomers can exhibit effects due to both attachment of the polystyrene to the alkyl-silica surface and to size exclusion. The retention volume, V_R, is then given (ref. 1, p. 25) as:

$$V_R = V_0 + K_{sec}V_i + K_{1c}V_s$$

$$= V_{sec} + K_{1c}V_s \quad (1)$$

Here, V_0 and V_i refer to the intra-column volumes (ml) of mobile phase contained within (i) or without (0) the pores of the packing. K_{sec} is the size-exclusion-chromatography (SEC) distribution constant, K_{1c} is the reversed-phase-retention distribution constant —equal to $(X)_s/(X)_m$, where $(X)_i$ refers to the concentration of solute X in the stationary phase (s) or mobile phase (m), and V_s is the volume of the stationary phase within the column. The quantity V_{sec} is then the retention volume of a solute that is not retained within the reversed-phase layer (K_{1c} equal zero).

We can also define the capacity factor, k', for reversed-phase retention as the total amount of solute in the stationary phase divided by the total amount of solute in the mobile phase (having a volume of $V_0 + V_i = V_m$). Appendix I shows that k' is given as:

$$k' = (V_R - V_{sec})/V_{sec}$$

$$= (t_R - t_{sec})/t_{sec} \quad (2)$$

Here, t_R is the retention of a band and t_{sec} is the value of t_R with no reversed-phase retention ($K_{1c} = 0$). In Appendix I we further show that:

$$K_{1c}V_s = k'V_{sec} \tag{3}$$

It is possible to measure k' from eqn. 2 and experimental values of t_R and t_{sec}*. V_{sec} is given as Ft_{sec}, where F is the flow-rate (ml/min) of mobile phase through the column. Thus we can measure values of $K_{1c}V_s$ as a function of experimental conditions: mobile phase composition, column-packing material, temperature, solute composition, etc. These values of $K_{1c}V_s$ can be used to construct a model for the retention of large molecules in reversed-phase systems, and the model can in turn be used to predict experimental conditions for the optimum separation of any sample.

In this study we will focus on the effects of mobile phase composition and the pore diameter/surface area characteristics of the column packing as these determine the retention of polystyrene molecules of varying molecular weight. We will use the general notation

$$K_{1c} = (K_i)_j$$

to define values of K_{1c} as a function of (a) the volume fraction, φ, of tetrahydrofuran (THF) in the mobile phase (equal to i) and (b) the pore diameter (nm) of the column packing (equal to j). Thus, the quantity $(K_{0.6})_{15}$ refers to a value of K_{1c} determined with 60% (v/v) THF–water ($\varphi = 0.6$) and a packing of 15-nm pore diameter. We will see that K_{1c} varies with mobile phase composition or the value of φ. However, for packings such as those of Table I where the bonding chemistry is the same and the surface structure (C_{18}) should therefore be similar as packing surface area and pore diameter are varied, K_{1c} is expected to be constant for a given solute X, a given value of φ, and different column packings. We will see that this is the case for the present systems.

To a first approximation, the actual volume of the stationary phase, V_s, can be assumed to be given by

$$V_j = d_{18}(SA)_j \tag{4}$$

where V_j refers to the value of V_s for a packing of pore diameter j (*e.g.* V_{100} refers to V_s for a 100-nm-pore packing), d_{18} is the effective thickness of the C_{18} bonded-phase layer, and $(SA)_j$ is the surface area of the same (100-nm) packing. The *effective* volume of the stationary phase may be less than this value, however, because of the inability of large molecules to enter small pores which contain some fraction of the total stationary phase. This gives rise to two limiting models for reversed-phase retention of large solute molecules: (a) the "softball" model and (b) the "hardball" model, as illustrated in Fig. 1. It is convenient to describe these two models in terms of analogous descriptions of the SEC retention of large molecules on packings of varying pore size.

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* Values of t_{sec} will vary with mobile phase composition, φ, but this variation is small and its effect on calculated values of k' is essentially negligible. We have used values of t_{sec} for 100% (v/v) THF (as tabulated in Table II) in all calculations of k' via eqn. 2.

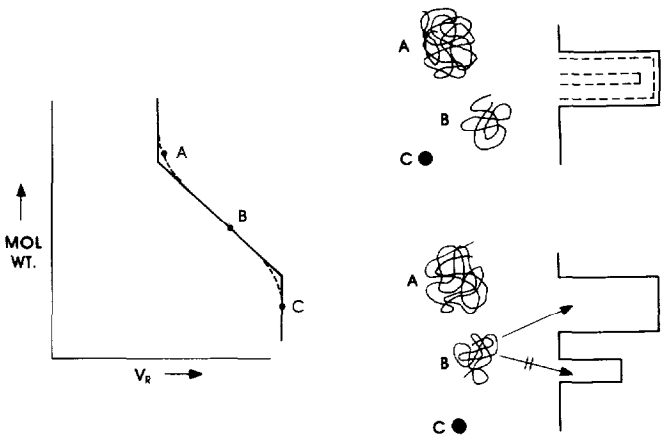

Fig. 1. Illustration of "softball" and "hardball" models of size-exclusion and reversed-phase retention. Softball model assumes compounds A, B, and C have access to particle pore shown on right. Dashed lines within pore indicate volume available to center of solute molecule for compounds B and C (see Fig. 14.10 of ref. 1).

The softball model of Fig. 1 assumes that SEC separation can occur within pores of a single diameter (Yau–Bly single-pore model of ref. 1, see discussion of their Fig. 14.10). Thus, the center of a molecule such as B is sterically restricted (on average) to the dashed region in the center of the pore. The center of a small molecule such as C is restricted to a larger region (also shown in Fig. 1), and C will therefore spend more time within the pore of the particle. While a large molecule such as A appears to be totally excluded from the pore on the basis of its SEC calibration plot (left-hand side of Fig. 1), it can nevertheless enter the pore by partial unfolding; however, entropic restriction which the molecule would thereby experience results in very few molecules of A within the pore. However, once inside the pore, a molecule such as A can be retained by reversed-phase attachment to the stationary phase (C_{18} layer), and it then has access to the entire stationary phase volume, V_s. Thus, for a given solute molecule X separated on column packings of pore diameter j and k with the same mobile phase i

$$\text{(softball)} \qquad (K_i)_j V_j/(K_i)_k V_k = V_j/V_k \tag{4a}$$

because K_i is the same for X when φ is constant and the column packing is varied. Moreover, the value of V_j/V_k will be the same for different solutes X and the same two packings j and k.

The hardball model of Fig. 1 assumes that SEC separation occurs within pores of varying diameters. Thus, molecule A is totally excluded from all pores of the packing, while compound B can enter the wide pores but not the narrow pores of the packing. Applying the hardball model to reversed-phase retention, we except that pores which are inaccessible for SEC retention ($K_{sec} = 0$) will also be inaccessible for reversed-phase retention. To a first approximation, for pores of not too different diameter, the *effective* or accessible stationary phase volume will then be equal not to V_s for a given solute, but to $V_s K_{sec}$. Thus, for pores too small for the solute to enter,

K_{sec} is zero and no retention by reversed phase will occur. This suggests for the hard-ball model that eqn. 4a can be rewritten as:

$$\text{(hardball)} \qquad (K_i)_j V_j/(K_i)_k V_k = (V_j/V_k)(K_j/K_k)_{sec} \tag{4b}$$

Here, K_j and K_k are the K_{sec} values for compound X and packings j and k, respectively.

The softball and hardball models lead to greatly different predictions of solute retention as a function of solute molecular size and packing pore diameter. Thus, values of $K_{1c}V_s$ are roughly proportional to values of k' (eqn. 2, where V_{sec} varies by less than two-fold), while the factor $(K_j/K_k)_{sec}$ of eqn. 4b can vary from zero to one. Comparison of experimental retention data with corresponding values predicted by each model can then yield a conclusion as to which model best describes a given experimental system.

In considering the application of the softball and hardball models for the packings of Table I and polystyrenes as solutes, two observations should be made. First, the pore structure of these packings is created by fusing together micro spheres of constant size. This then yields pores of constant size as described in the Yau–Bly single-pore model of SEC[1]. Second, if the random-coil structure of the polystyrene molecule is extended or unfolded, the cross-section of the resulting molecule is small enough to easily allow penetration of the solute into the smallest pores present in the packings studied by us (6-nm pores, see Table I). Thus all of the conditions implied or required in the softball model are met in the case of polystyrenes and the present packings. This might not be true for other macromolecules (*e.g.*, proteins) or for other packing materials.

Other relationships. For small molecules in reversed-phase systems, their k' values usually vary with the volume fraction φ or organic solvent in the mobile phase as (*e.g.*, ref. 16):

$$\log k' = \log k_w - S\varphi \tag{5}$$

Here, k_w is the extrapolated value of k' for water as mobile phase, and S is the slope of the $\log k'$ *vs.* φ plot. Eqn. 4 is empirical, but it is adequately precise over the usual range in φ where k' is of interest ($0.5 < k' < 10$), and especially for organic solvents such as methanol and acetonitrile.

Schoenmakers *et al.*[10,11] have argued on theoretical grounds that a more precise formulation is:

$$\log k' = A\varphi^2 + B\varphi + C \tag{5a}$$

We will see here for polystyrenes eluted in THF–water mobile phases that eqn. 5 seems to hold over wide ranges in φ. For different packings as in the present study where surface area and pore size are varied but surface chemistry is held constant, it then follows from eqn. 5 (and the discussion of eqn. 4a) that values of S should be constant for different packings and a given solute. We will show that this is the case.

For small, repeat-unit solutes in reversed-phase systems it is usually observed that the Martin equation[17] is obeyed:

$$\log k' = A + Bn \tag{5b}$$

Here, k' is a value for an oligomer containing n repeat-units (same mobile phase and packing). A and B are constants for the given HPLC system and a particular oligomer series. Eqn. 5b has been shown to hold for the normal-phase separation of polystyrenes with $10 \leqslant n \leqslant 30$ (ref. 18, p. 582). Comparison of experimental data with eqn. 5 allows additional insight into the retention process, particularly as n is varied over wide limits.

Gradient elution relationships

Several studies have shown (*e.g.*, refs. 10, 16) that the constant S in eqn. 5 often increases with solute molecular weight, so that the dependence of k' on φ can be quite steep for large macromolecules such as proteins (*e.g.*, ref. 15). This in turn often makes gradient elution the preferred separation procedure in reversed-phase systems. If eqn. 5 is obeyed approximately over the usual range in k' ($1 \leqslant k' \leqslant 10$), and if linear gradients are employed, the value of k' at the column inlet during gradient elution will be given as:

$$\log k_i = \log k_0 - b(t/t_0) \tag{6}$$

Here, k_0 is the value of k_i at the beginning of separation ($t = 0$), t is the time after sample injection and the simultaneous beginning of the gradient, and b is a gradient steepness parameter given by[14]:

$$b = \Delta\varphi \; S \; t_0/t_G \tag{6a}$$

The quantity $\Delta\varphi$ refers to the change in φ during the gradient, S is for the solute in question, and t_G is the time during gradient elution while φ is varying.

Retention in gradient elution separations where eqn. 6 applies with constant values of b for all solutes (so-called linear-solvent-strength or LSS separation) is given as:

$$t_g = (t_0/b) \log(2.3 \; b \; k_0 + 1) + t_0 \tag{7}$$

Eqn. 7 assumes no lagtime, t_L, between mixing of the mobile phase and entry of the mobile phase into the column, and ignores SEC effects of the type under discussion. In Appendix II it is shown for the case of significant solute exclusion ($K_{sec} < 1$) and significant values of t_L that*:

$$t_g = (t_0/b)\log[2.3bk_0(t_{sec}/t_0) + 1] + t_{sec} + t_L \tag{8}$$

It has also been shown[14] that LSS separations are generally optimum in terms of providing similar resolution throughout the gradient chromatogram. The optimum value of b for such separations, analogous to an optimum k' value of 2–5 in isocratic separation, is $0.2 \leqslant b \leqslant 0.5$.

* Eqn. 8 assumes that k_0 is large so that the solute band does not migrate significantly during passage of the volume, t_L, of starting mobile phase through the band center, see Appendix II.

It is possible to use eqn. 8 to derive the isocratic parameters k_0 and S for a given solute–column combination, as an alternative to determining these quantities directly via isocratic separations. This is of particular interest for the case of macromolecules with large values of S, and specifically for the polystyrenes studied here. The reason is that with large values of S, there is only a narrow range in φ corresponding to the reasonable values of k'. This has been noted by others (*e.g.*, ref. 19), who find as we did that isocratic elution appears to give either total retention of a solute band or elution with the solvent front. This is illustrated for our system in Fig. 2, where the elution of a 50,000-mol.wt. polystyrene is shown from a 30-nm-pore packing, using 83, 85 and 87% (v/v) THF–water. No observable band results for 83% (v/v) THF, while immediate elution occurs for 87% THF. Only at 85 ± 1% (v/v) THF are reasonable values of k' observed.

The approach used here to derive values of k_0 and S from gradient runs is as follows. With otherwise identical conditions ($\Delta\varphi$, gradient shape, sample, column, temperature, flow-rate, etc.), gradient separations are carried out using two different values of t_G (t_{G1}, t_{G2}), so as to vary the value of b (eqn. 6a). This in turn yields values of t_g for the two separations, and corresponding values of b: t_{g1}, b_1 (t_{G1}); t_{g2}, b_2 (t_{G2}). Now a value of b_1 is estimated, since S is not initially known. Eqn. 8 then permits the calculation of a corresponding value of k_0 (values of t_0, t_{sec} and t_L will be known initially). A value of b_2 for the second run is next calculated from the estimated value of b_1: $b_2 = (t_{G1}/t_{G2})\, b_1$, from eqn. 6a. Resulting values of b_2 and k_0 then permit calculation of t_{g2} (eqn. 8), and this value is compared with the experimental value. Trial-and-error insertion of different estimates for b_1 into the latter calculation sequence finally allows a best fit of calculated and experimental values of t_{g2}. This procedure (using a computer) can provide a rapid determination of best values of b_1, b_2 and k_0 from experimental values of t_{g1} and t_{g2}. Eqn. 6a then yields a corresponding value of S.

RESULTS AND DISCUSSION

Retention of individual polystyrene oligomers

The reversed-phase separation of lower-molecular-weight polystyrene samples as in the present study is capable of resolving individual oligomers according to the

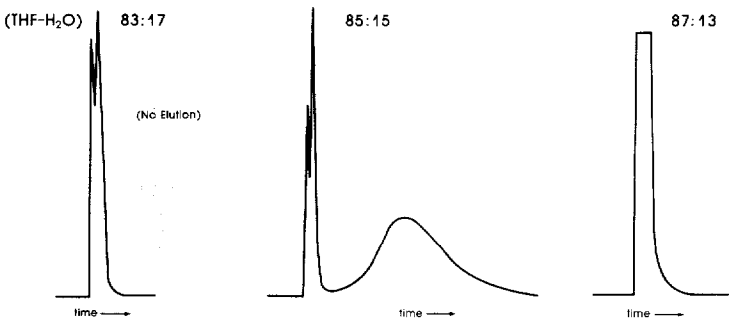

Fig. 2. Isocratic elution of 50,000-mol.wt., polystyrene standard from 30-nm-pore packing by mobile phases of varying tetrahydrofuran concentration.

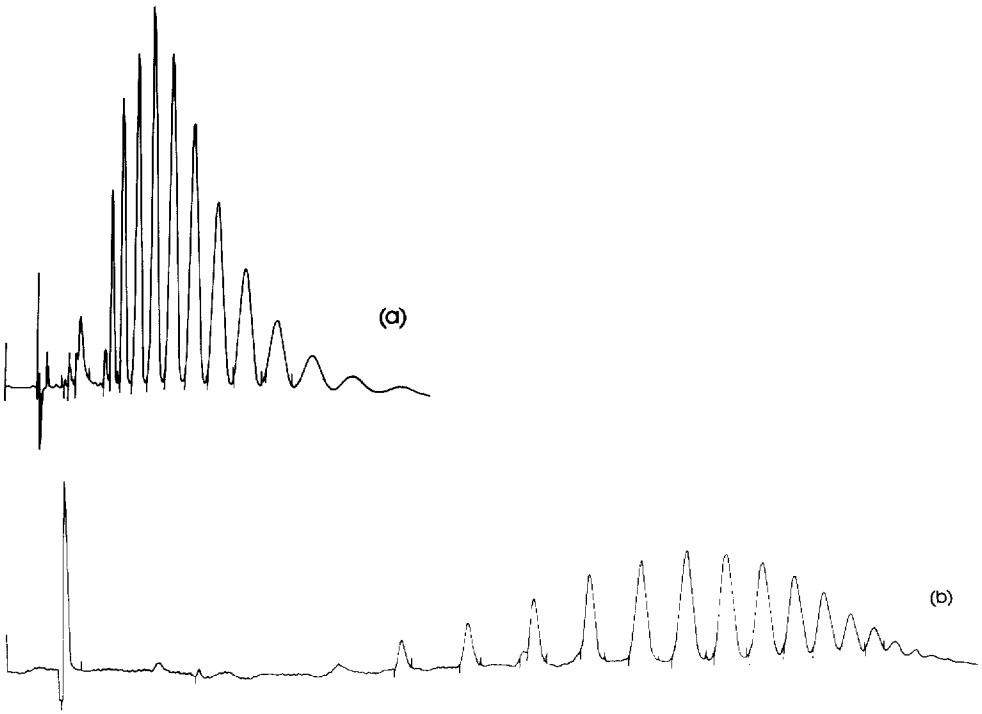

Fig. 3. Separation of 800-mol.wt. polystyrene by isocratic (a) and gradient elution (b) from 6-nm-pore C_{18} column. Conditions: (a) 72% (v/v) THF–water; (b) linear gradient, 60% (v/v) THF–water to 90% (v/v) THF–water in 20 min.

number n of repeating units in the molecule. This is illustrated in Fig. 3 for both the isocratic (a) and gradient elution (b) separation of an 800-mol.wt. sample. Such separations therefore allow the direct measurement of isocratic or gradient retention times (t_R, t_g). We have carried out isocratic separations over a broad range of THF–water compositions (values of φ), using the four reversed-phase packings of Table I and the 800-mol.wt. polystyrene of Fig. 3 ($2 \leqslant n \leqslant 12$). Values of k' were next calculated from values of t_R, using eqn. 2 and values of t_{sec} interpolated from SEC calibration plots summarized in Table II. Table III summarizes resulting values of log k' as a function of n, φ and packing type. Plots of log k' *vs.* φ (data of Table III) were observed to be linear for every oligomer and column packing studies. This is illustrated in Fig. 4 for the 15-nm-pore packing. That is, eqn. 5 describes these data, rather than eqn. 5a. The ability of eqn. 5 to correlate values of k' and φ for the present system is further shown in Table IV, where derived values of S for each oligomer and column packing are summarized. Since the stationary phase (C_{18}) is the same for each of these packings, S should likewise be constant for a given oligomer and the four packings, and this is observed ($\pm 2.4\%$ in Table IV). This represents a further test of eqn. 5 *vs.* 5a, since the latter equation predicts that values of S should increase for packings of smaller surface area and V_s, because these packings involve smaller values of φ (see Table III). Table V summarizes derived (average) values of log ($K_{0.6}V_s$), obtained by first calculating k' for φ equal 0.6 from eqn. 5 and the data of Table III,

TABLE II

SIZE-EXCLUSION CHROMATOGRAPHY CALIBRATION OF PACKINGS OF TABLE I

Retention times, t_{sec}, for tetrahydrofuran as mobile phase; flow-rate 2.0 ml/min.

Sample (polystyrene) mol.wt.	t_{sec} *(min)*			
	6 nm	*15 nm*	*30 nm*	*100 nm**
92**	1.24	1.41	1.47	0.76
800	1.05	1.27	1.42	
2000	0.97	1.19	1.39	
4000	0.92	1.13	1.36	
9000	0.87	1.06	1.32	
17,500	0.83	0.98	1.24	
50,000	0.83	0.92	1.12	
100,000	0.83	0.88	1.07	
233,000	0.83	0.90	0.95	
Exclusion	0.83	0.89	(0.89)	0.76

* Pellicular particle; does not exhibit SEC effects.

** Styrene.

then using eqn. 3 to calculate values of $K_{0.6}V_s$. The quantity $K_{0.6}$ refers to the value of K_{1c} for $\varphi = 0.6$. We will return to the significance of these data (Table V) in a later section.

Retention of higher-molecular-weight polystyrenes

As illustrated by Fig. 2 and the accompanying discussion in the Theory section, the isocratic measurement of values of k_0 and S presents certain difficulties in the case of compounds or samples with large values of S (large molecules such as the 50,000-mol.wt. polystyrene of Fig. 2). We have therefore studied the measurement of these parameters by means of gradient elution. A more complete discussion of this technique will be published elsewhere. Table VI summarizes the experimental data used in this connection.

Using the approach described in the Theory section, values of b (b_5) for t_G equal 5 min were derived from experimental t_g values for different pairs of t_G values. Constancy in these b_5 values for different pairs of t_g values is one check on the overall reliability of this procedure, and is summarized in Table VII for one of the four packings (15-nm pore). In addition to values of b_5 calculated as described earlier, "best" values of b_5 were also obtained by least-squares fitting of all data points to a single value of b_5. These results are also included in Table VII. For the lower-molecular-weight polystyrenes (mol.wt. $\leqslant$ 9000), the various values of b_5 obtained for each sample are reasonably consistent. However, for higher-molecular-weight samples, values of b_5 show wide variations —implying less accuracy for their determination in this manner. An analysis that will be presented elsewhere shows that the present procedure is less reliable when the resulting b values are greater than about 2, which is the case for the data of Table VII for 17,500- and 50,000-mol.wt. samples. In this case, the best approach is to use several data points (t_g values) with least-squares fitting. However, the corresponding reliability of these derived k_0 and S values will decrease with increasing values of S and k_0 (*i.e.*, increasing mol.wt. of the sample).

TABLE III

Log k' RETENTION DATA FOR POLYSTYRENE OLIGOMERS ($2 \leqslant n \leqslant 12$)

Volume fraction, φ, of tetrahydrofuran–water mobile phases varied for packings of different pore diameter (see Table I); values calculated from eqn. 2 with t_{sec} values interpolated from Table II.

Value of n	*6-nm-pore particle*				*15-nm-pore particle*					*30-nm-pore particle*				*100-nm-pore particle*
φ =	*0.70*	*0.72*	*0.75*	*0.78*	*0.65*	*0.68*	*0.70*	*0.72*	*0.75*	*0.63*	*0.65*	*0.68*	*0.70*	*0.53*
2	0.27	0.18	0.04	−0.10	0.30	0.17	0.08	−0.02	−0.16	−0.04	−0.13	−0.27	−0.34	
3	0.37	0.27	0.12	−0.03	0.41	0.27	0.17	0.07	−0.09	0.09	−0.02	−0.16	−0.25	−0.29
4	0.47	0.36	0.19	0.03	0.53	0.36	0.26	0.15	−0.02	0.20	0.09	−0.09	−0.17	−0.16
5	0.56	0.44	0.26	0.09	0.63	0.46	0.35	0.23	0.05	0.31	0.18	0.01	−0.10	−0.03
6	0.64	0.52	0.32	0.14	0.73	0.53	0.41	0.28	0.09	0.40	0.27	0.08	−0.03	0.10
7	0.73	0.59	0.39	0.19	0.82	0.61	0.48	0.34	0.14	0.49	0.35	0.15	0.03	0.23
8	0.80	0.66	0.44	0.24	0.91	0.68	0.54	0.40	0.19	0.57	0.43	0.21	0.09	0.36
9		0.73	0.50	0.28		0.76	0.61	0.46	0.24	0.65	0.50	0.28	0.15	0.48
10		0.79	0.55	0.33		0.83	0.67	0.52	0.29	0.73	0.57	0.34	0.20	0.60
11			0.60	0.36		0.90	0.74	0.57	0.31	0.80	0.63	0.40	0.25	0.69
12			0.65	0.41				0.63	0.38	0.88	0.71	0.45	0.30	

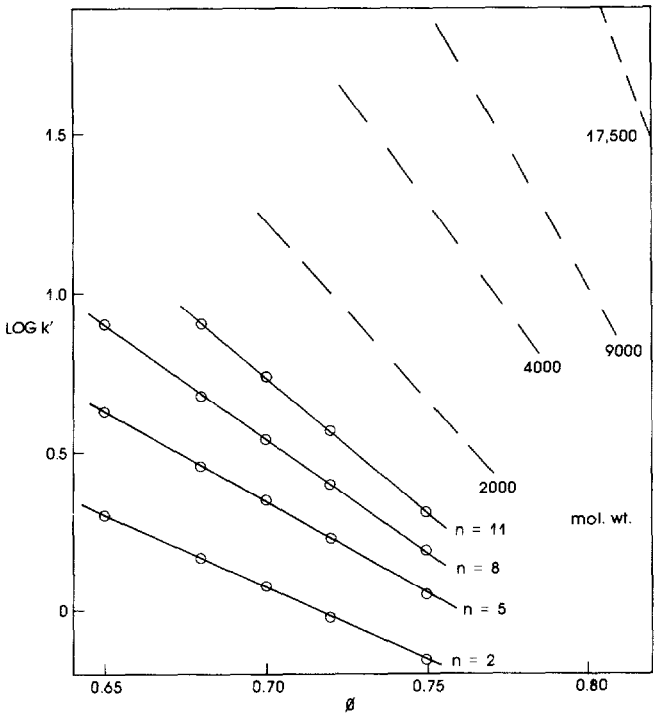

Fig. 4. Dependence of k' on mobile phase composition, φ, for different polystyrenes and 15-nm-pore column. ○—○, Oligomers of indicated n value (data of Table III); ———, polystyrene standards of indicated mol.wt. (gradient data).

TABLE IV

DEPENDENCE OF SAMPLE RETENTION ON MOBILE PHASE COMPOSITION FOR VARIOUS POLYSTYRENE OLIGOMERS AND DIFFERENT PACKINGS

Slopes S of log k' *vs.* φ plots (data of Table III).

Value of n	*S**			
	6 nm	*15 nm*	*30 nm*	*Av.*
2	4.64	4.60	4.40	4.55
3	5.04	5.07	4.83	4.98
4	5.50	5.46	5.30	5.42
5	5.93	5.95	5.76	5.88
6	6.31	6.24	6.13	6.23
7	6.72	6.61	6.50	6.62
8	7.08	6.98	6.81	6.96
9	7.37	7.34	7.17	7.29
10	7.77	7.70	7.48	7.65
11		8.03	7.81	7.92
12			8.30	8.30

* For indicated packings (pore size).

TABLE V

DERIVED VALUES OF $K_{0.6}V_s$

Oligomer data from Tables III and IV and eqns. 3 and 5; polymer standards (mol.wt. 2000–50,000) from Tables VI and VII and eqn. 8.

Sample	*log ($K_{0.6}V_s$) for indicated pore diameter*			
	6 nm	*15 nm*	*30 nm*	*100 nm*
$n = 2$	1.13	0.98	0.56	
3	1.27	1.12	0.70	−0.64
4	1.41	1.25	0.83	−0.54
5	1.54	1.38	0.94	−0.45
6	1.67	1.48	1.04	−0.33
7	1.79	1.59	1.14	−0.23
8	1.89	1.69	1.24	−0.13
9	2.00	1.79	1.33	−0.03
10	2.10	1.89	1.42	0.06
11		2.03	1.50	0.14
12			1.59	
2000*	3.20	2.74	2.43	1.23
4000	3.99	3.69	3.32	1.99
9000	5.27	4.91	4.60	3.42
17,500	8.17	8.05	7.63	6.34
50,000	14.83	14.65	14.52	13.41

* Polymer standards of indicated mol.wt.

Resulting values of S and k_0 for $\varphi = 0.6$ ($k_{0.6}$) from the gradient data of Table VI are summarized in Table VIII. Consider the S values first. We have already noted that the latter values will be less precisely determinable than for the case of the lower-molecular-weight oligomers of Table IV. This is confirmed by a comparison of the data of Table IV *vs.* Table VIII. Thus S values are expected to be constant for a given sample and different packings. This is true in both Tables IV and VIII, but the variation of S values is much greater in Table VIII than in Table IV: a C.V., of 15% in Table VIII *vs.* only 2.4% in Table IV. However, there are no obvious trends in S with change in packing in Table VIII, and we can therefore improve the reliability of resulting S values for a given sample by averaging the S values for each sample (last column in Table VIII).

With improved (averaged) S values, the data of Table VI can be used (eqn. 8) to obtain average (least-squares) values of k_0, which then permits the derivation of best values of $k_{0.6}(t_{sec}/t_0)$ as summarized in Table VIII. With the resulting data of Table VIII, it is possible to calculate t_g values corresponding to experimental values in Table VI (values in parentheses). The overall agreement of experimental and calculated t_g values in Table VI is ± 0.13 min (1 S.D.) for $6 \leqslant t_g \leqslant 127$ min. Thus, final (averaged) values of the retention parameters as in Table VIII yield excellent agreement between experimental and calculated t_g values for the data of Table VI. This means that prediction of gradient retention as a function of varying experimental conditions is possible with the present data base for polystyrenes and the present packings. Some further checks on the reliability of these retention parameters will be offered shortly.

TABLE VI

RETENTION TIMES, t_g, FOR VARIOUS POLYSTYRENE SAMPLES IN GRADIENT ELUTION FROM DIFFERENT PACKINGS

Calculated values in parentheses are from eqn. 8 with parameters of Tables II and VIII. t_L = 2.2 min.

Polystyrene mol.wt.	*6-nm pore**		*15-nm pore**				*30-nm pore***			*100-nm pore****	
t_G =	*10 min*	*40 min*	*5 min*	*10 min*	*40 min*	*80 min*	*5 min*	*40 min*	*160 min*	*5 min*	*40 min*
2000	9.38	23.80	6.64	8.96	19.80		7.24	27.00		6.32	25.8
	(9.64)	(23.76)	(6.44)	(8.82)	(19.83)		(7.19)	(27.06)		(6.32)	(25.82)
4000	9.87	26.45	6.81	9.69	24.50		7.41	29.52		6.58	28.4
	(10.04)	(26.39)	(6.80)	(9.71)	(24.46)		(7.36)	(29.54)		(6.56)	(28.41)
9000	10.16	28.75	6.92	10.05	26.90		7.55	32.05		6.85	31.45
	(10.31)	(28.67)	(6.85)	(10.02)	(26.93)		(7.51)	(32.16)		(6.84)	(31.47)
17,500	10.33	29.74	6.95	10.20	29.09	54.05	7.62	33.87	119.4	7.08	33.7
	(10.25)	(29.81)	(6.87)	(10.29)	(29.52)	(53.79)	(7.49)	(33.80)	(119.47)	(7.00)	(33.68)
50,000	10.35	30.75	6.95	10.24	30.27	56.88	7.66	35.01	127.0	7.22	35.8
	(10.27)	(30.75)	(6.76)	(10.26)	(30.53)	(56.79)	(7.42)	(35.02)	(126.97)	(7.17)	(35.79)

* Gradient from 60 to 100% THF.
** Gradient from 40 to 100% THF.
*** Gradient from 20 to 100% THF.

TABLE VII

CONSISTENCY OF b VALUES DERIVED FROM DIFFERENT PAIRS OF t_g VALUES (DATA OF TABLE VI) FOR 15-nm-PORE PACKING

*Sample**	t_{g1}/t_{g2} *(min)*	b_5**
2000	5/40	1.05
	10/40	1.14
	Best fit***	1.09
4000	5/40	1.53
	10/40	1.59
	Best fit	1.56
9000	5/40	1.81
	10/40	1.93
	Best fit	1.87
17,500	5/80	2.9
	10/80	3.9
	40/80	7.2
	Best fit	4.2
50,000	5/80	3.6
	10/80	6.4
	40/80	12.4
	Best fit	6.4

* Polymer standards of indicated mol.wt.
** Value of b for 5-min run.
*** Least squares fit for best value of b_5.

TABLE VIII

VALUES OF S AND $k_{0.6}(t_{sec}/t_0)$* DERIVED FROM GRADIENT ELUTION DATA OF TABLE VI FOR DIFFERENT POLYMER SAMPLE–PACKING COMBINATIONS

Sample (mol.wt.)	S ($\log k_{0.6}t_{sec}/t_0$)**				
	6-nm pore	*15-nm pore*	*30-nm pore*	*100-nm pore*	*Av.*
2000	14.3	9.7	10.0	11.3	11.3
	(2.81)	(2.29)	(1.96)	(1.05)	
4000	16.4	13.8	11.9	12.5	13.7
	(3.60)	(3.24)	(2.75)	(1.81)	
9000	22.5	16.6	15.1	17.2	17.8
	(4.88)	(4.46)	(4.13)	(3.24)	
17,500	24.2	34.6	27.2	28.9	28.7
	(7.78)	(7.60)	(7.16)	(6.16)	
50,000	38.7***	56.7	52.5	48.6	52.6
	(14.44)	(14.20)	(14.05)	(13.23)	

* Equal also to $K_{0.6}(V_s/V_m)$; eqn. 3, with $V_{sec}/V_m = t_{sec}/t_0$.

** Values of S calculated from data of Table VI using eqns. 6a, 8; values of $\log [k_{0.6}(t_{sec}/t_0)]$ from best (least-squares) fit of data of Table VI using average S values above.

*** Value excluded from average because of large deviation from mean.

Variation in V_s among different column packings

Eqn. 3 and the data of Tables III, IV, VI and VIII allow the calculation of best values of $K_{0.6}V_s$ for each sample (oligomers and polymer standards) and each column packing. Using the 100-nm-pore packing as reference, with V_s defined equal to V_{100} for that packing, eqn. 4a (softball model) predicts that the ratio V_s/V_{100} is given as

$$V_s/V_{100} = (K_{0.6}V_s)/(K_{0.6}V_{100}) \tag{9}$$

since $K_{0.6}$ for a given solute will be the same for various packings. Moreover, this ratio V_s/V_{100} will be constant for all solutes. This prediction of the softball model is tested in Table IX, and seen to be verified. Thus, for all polystyrene solutes, values of $\log(V_s/V_{100})$ are equal respectively to 1.33 ± 0.06 (30-nm), 1.75 ± 0.11 (15-nm) and 1.97 ± 0.06 (6-nm). Values for the 50,000-mol.wt. polystyrene are excluded from this comparison and will be discussed separately.

The above discussion of Table IX supports the softball model and the concept that the entire stationary phase is available for retention of polystyrenes that are at least as large 17,500 mol.wt. If the hardball model were instead appropriate, we would have from eqn. 4b for V_s/V_{100}:

$$V_s/V_{100} = (K_{0.6}V_s)/(K_{0.6}V_{100})\ (K_{100}/K_{sec}) \tag{9a}$$

Here, the quantity V_s/V_{100} should again be constant for various solutes. The last column of Table IX shows this prediction of the hardball model for a comparison of the 6-nm *vs.* 100-nm-pore packings. The resulting values of V_6/V_{100} are seen to vary regularly with increasing solute molecular weight, and to become infinite for the 17,500-mol.wt. polystyrene. The next to last column of Table IX, for the same com-

TABLE IX

DERIVED VALUES OF (V_s/V_{100}) FROM DATA OF TABLES III AND VI

See text for details.

Sample	*log* (V_s/V_{100})			*log* (V_6/V_{100}) *(hardball model, eqn. 9a)*
	30-nm pore	*15-nm pore*	*6-nm pore*	
$n = 3$	1.34	1.76	1.91	2.00
4	1.37	1.79	1.95	2.09
5	1.39	1.83	1.99	2.17
6	1.37	1.81	2.00	2.22
7	1.37	1.82	2.02	2.27
8	1.37	1.82	2.02	2.30
9	1.37	1.82	2.03	2.34
10	1.36	1.83	2.04	2.37
11	1.36	1.89		
2000*	1.20	1.51	1.97	2.44
4000	1.33	1.70	2.00	2.66
9000	1.18	1.49	1.85	2.86
17,500	1.29	1.71	1.83	∞
Average**	1.33	1.75	1.97	
	± 0.06	± 0.11	± 0.06	
50,000	1.11	1.24	1.42	∞
$\bar{V}_s/\bar{V}_s$	0.60	0.31	0.28	

* Polymer standards of indicated mol.wt.

** Average of values for each packing and all samples except 50,000-mol.wt. polystyrene.

TABLE X

CONSTANCY OF K_{1c} FOR VARIOUS SOLUTES ON DIFFERENT PACKINGS

Solute	*log* $(K_{0.6}V_{100})$*				
	6-nm pore	*15-nm pore*	*30-nm pore*	*100-nm pore*	*Av.*
$n = 2$	-0.84	-0.77	-0.77	–	-0.79
3	-0.70	-0.63	-0.63	-0.64	-0.65
4	-0.56	-0.50	-0.50	-0.54	-0.52
5	-0.43	-0.37	-0.39	-0.45	-0.41
6	-0.30	-0.27	-0.29	-0.33	-0.30
7	-0.18	-0.16	-0.19	-0.23	-0.19
8	-0.08	-0.06	-0.09	-0.13	-0.09
9	0.03	0.04	0.00	-0.03	0.01
10	0.13	0.14	0.09	0.06	0.10
11	–	0.28	0.17	0.14	0.20
12	–	–	0.26	–	0.26
2000**	1.23	0.99	1.10	1.23	1.11 ± 0.12
4000	2.02	1.94	1.99	1.99	1.98 ± 0.03
9000	3.30	3.16	3.27	3.42	3.29 ± 0.11
17,500	6.20	6.30	6.30	6.34	6.28 ± 0.06
50,000	12.86	12.90	13.19	13.41	$(13.1) \pm 0.2$

* From eqn. 10.

** Polystyrene standards of indicated molecular weight.

parison of the *softball* model, shows excellent constancy of values of V_6/V_{100}. *This provides dramatic proof that the softball model applies to this system, and the hardball model does not.*

Average values of $K_{0.6}V_{100}$

Further averaging of retention data for the various polystyrene solutes can be achieved by calculating values of $K_{0.6}V_s$ for the 100-nm packing from data for each packing. Thus if $K_{0.6}$ for each solute is constant for all packings, the same value of $K_{0.6}V_{100}$ should result from the relationship:

$$K_{0.6}V_{100} = (K_{0.6}V_s)(V_{100}/V_s) \tag{10}$$

Values of (V_{100}/V_s) from Table IX can be used with previously determined values of $K_{0.6}V_s$ to yield values of $K_{0.6}V_{100}$ from eqn. 10. Resulting values of $\log(K_{0.6}V_{100})$ are summarized in Table X. For each of the oligomers, the overall agreement of values of $\log(K_{0.6}V_{100})$ for each compound is ± 0.03 units (1 S.D.). The corresponding agreement of the polystyrene standards (mol.wt. 2000–17,500) is somewhat poorer but still reasonable (± 0.09 units). For elution of each of these compounds at a given k' value, an error in $\log(K_{0.6}V_{100})$ as in Table X corresponds to a corresponding error in φ, equal to (error)/S. In these terms it is seen that the error for the oligomers is about $0.03/6.4 = 0.5\%$ (v/v) in φ. Similarly, the error for the polystyrene standards varies from 0.2–1.0% (v/v) and averages 0.6% (v/v) —about the same error as for the oligomers. For gradient separations, we will show elsewhere that the error in $K_{0.6}$ expressed as %(v/v) THF is the more significant number.

Correlation of values of V_s *with packing surface area*

Eqn. 4 suggest that the quantity (V_s/V_{100}) as tabulated in Table IX should be related to the surface area within the column (last column of Table I) as:

$$V_j/V_{100} = (\mathrm{SA})_j/(\mathrm{SA})_{100} \tag{11}$$

Here, V_j equals V_s for different packings j, and $(\mathrm{SA})_j$ and $(\mathrm{SA})_{100}$ are total column surface areas for packing j and for the 100-nm-pore packing. This relationship is tested in Fig. 5, where V_s/V_{100} is plotted *vs.* the corresponding surface area ratio. The data points for the smaller-surface-area (larger-pore) packings fall generally close to the expected 45° line (solid curve in Fig. 5), but there is significant deviation of the narrower-pore, higher-surface packings. These deviations are not unexpected, and probably arise from the combination of small micro-particles which define the pore structure plus the bulky C_{18} bonded phase. Thus, the "true" surface area of the bonded-phase packings may not closely approximate the surface area of the underlying silica for narrow-pore packings.

Isocratic measurements of polystyrene-standard retention times

The reproducibility of $\log(K_{0.6}V_{100})$ values of Table X for the various polystyrene standards suggests that these values are accurate within about ± 0.1 unit for the polystyrenes of molecular weight 17,500 and lower, and about ± 0.2 units for the 50,000-mol.wt. polystyrene standard. With the data of Table X it is possible to es-

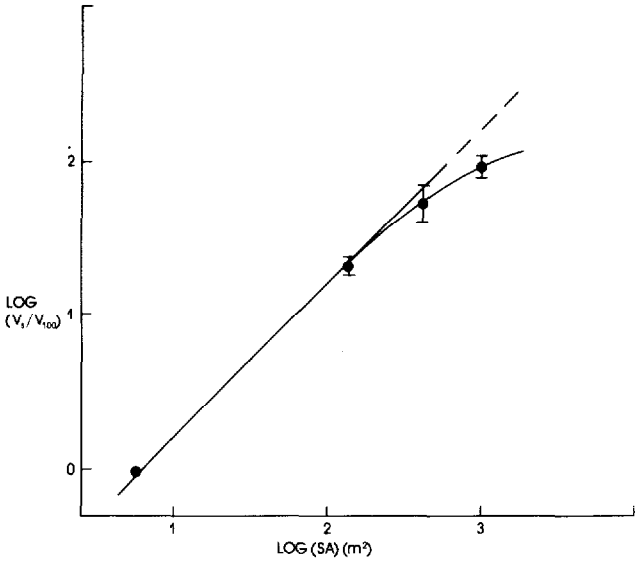

Fig. 5. Dependence of stationary phase volume, V_s, on silica surface area, SA.

timate values of k' for various polystyrenes and values of φ, and for the various packings of Table I. We have carried out isocratic measurements in a few selected cases for comparison with these values.

The results of this study are summarized in Table XI. For the 2000-mol.wt. sample, the agreement between experimental (isocratic) and calculated (gradient) values is ± 0.10 unit (1 S.D.), and for the 50,000-mol.wt. sample, ± 0.4 units. While the agreement for the latter sample is somewhat poorer then expected, the 2000-mol.wt. sample is in line with the data of Table X. The improvement of the present

TABLE XI

COMPARISON OF ISOCRATIC k' VALUES WITH CORRESPONDING VALUES CALCULATED FROM GRADIENT DATA OF TABLE VIII

Packing	*Solute**	φ	*log k'*	
			*Exptl.***	*Calc.****
6-nm	50,000	0.87	0.53	0.41
15-nm	2000	0.73	0.89	0.89
		0.75	0.76	0.67
		0.78	0.36	0.33
		0.80	0.18	0.10
		0.85	−0.31	−0.47
	50,000	0.85	1.15	1.23
		0.86	0.28	0.70
		0.87	−0.45	0.17

* Polystyrenes of indicated mol.wt.

** Isocratic value.

*** Calculated from gradient data of Table VIII.

procedure for extracting isocratic retention data from gradient elution experiments will be discussed elsewhere.

Comparisons of retention of large and small molecules in reversed-phase systems

It is interesting to compare the behaviour of the presently studied polystyrenes with that of smaller molecules in reversed-phase systems reported earlier. Schoenmakers *et al.*[10] have shown that in THF–water mobile phases, the values of S increase regularly with solute retention. Their data can be reexpressed as

$$S = 3.53 + 2.21 \log k_{0.6} \tag{12}$$

for a packing of presumably 10-nm pores. Using eqn. 11, eqn. 12 can be rewritten for 100-nm-pore packing as in the present study

$$S = 6.47 + 2.21 \log(k_{0.6})_{100} \tag{12a}$$

where $(k_{0.6})_{100}$ is calculable from eqn. 3 and values of $K_{0.6}V_{100}$ in Table X. Fig. 6 plots experimental values of S from the present study *vs.* values of $\log(k_{0.6})_{100}$, with the solid curve being drawn through the experimental data points. The dashed curve of Fig. 6 corresponds to the predicted dependence from Schoenmakers study of small molecules (eqn. 12a), which consisted mainly of benzene derivatives. In view of the scatter of points in the Schoenmakers correlation *vs.* eqn. 12[10], the agreement between these two studies is reasonably good and suggests that large molecules such as these polystyrenes behave similarly to what would be expected by the extrapolation of small-molecule behavior.

The Martin equation (eqn. 5) is tested in Fig. 7 for retention of the various polystyrenes on the 100-nm-pore packing with $\varphi = 0.60$ (data of Table X). The number of repeating units represented in this study ranges from 2 to 470, and log k'

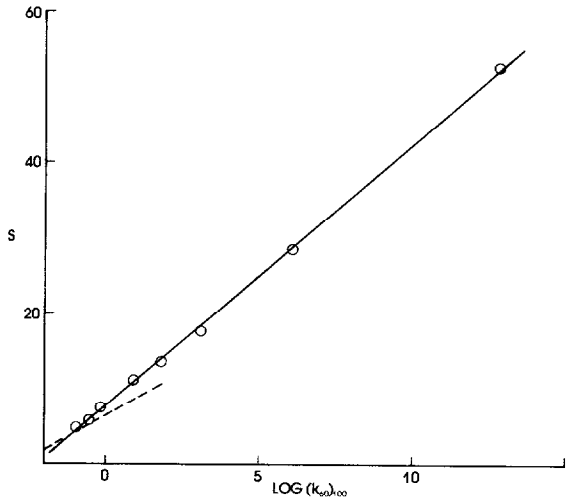

Fig. 6. Variation of S with solute retention. O—O, Polystyrenes from present study; ——, small molecules from ref. 10.

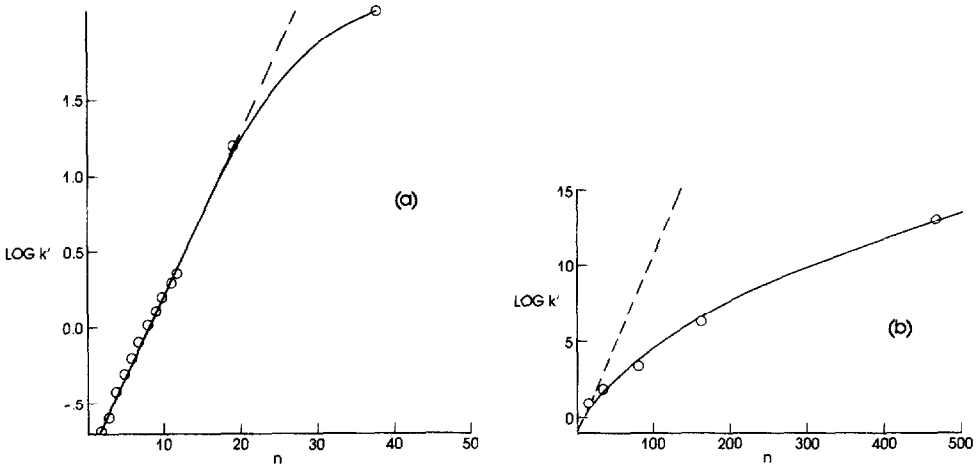

Fig. 7. Verification of the Martin equation for polystyrenes of varying molecular weight: (a) $2 \leqslant n \leqslant 40$; (b) $19 \leqslant n \leqslant 470$; 100-nm-pore packing, 60% (v/v) THF–water (Table X, eqn. 3).

ranges from -0.7 to 13.2. Thus, a more extended test of this relationship (eqn. 5) is possible than has been attempted previously. Fig. 7 shows two overlapping plots on different scales: $2 \leqslant n \leqslant 38$ in Fig. 7a, and $19 \leqslant n \leqslant 470$ in Fig. 7b. Examination of these data shows adherence to the Martin equation for $n \leqslant 19$, but a bending off of the straight line plot from lower n values when n exceeds about 20. A variety of causes for this behavior can be cited as possibilities: collapse of the extended (random-coil) configuration of the larger polystyrenes in the mobile phase due to hydrophobic effects, entropic exclusion of larger polystyrenes from the surface layer due to crowding effects, etc. We will examine these effects further in a later communication*.

Temperature and flow-rate effects

Studies similar to ours for the *normal-phase* retention of polystyrenes and other polymers have been reported[20] to show anomalous flow-rate and temperature effects. The latter observations suggest that retention of large molecules such as these may not reflect the usual equilibrium condition within the column that is observed for small molecules in most cases. We have therefore carried out preliminary observations of these effects in our system. The effect of varying flow-rate on retention was studied for the 50,000-mol.wt. polystyrene on the 6-nm packing at two temperatures, as summarized in Table XII.

Concerning the dependence of k' on flow-rate, the data of Table XII suggest that in our system a molecule as large as the 50,000-mol.wt. polystyrene does not exhibit a significant dependence of k' on flow-rate —suggesting that equilibrium is

* A reviewer has noted that higher-molecular-weight polystyrenes are insoluble under the conditions of initial gradient elution from the column (*i.e.*, at low φ values) and questions whether this might not affect the preceding anomalies noted for the 50,000-Dalton polystyrene sample. It is true that the latter sample is essentially insoluble in the 20–60% (v/v) THF–water solutions used as initial mobile phases in Table VI. However, this has no effect on the final retention data, inasmuch as the samples can only migrate when their k' values in the mobile phase are reduced to a value of about 10 (see discussion of ref. 14). Under conditions of $k' \leqslant 10$, the samples are adequately soluble in the mobile phase.

TABLE XII

FLOW-RATE AND TEMPERATURE EFFECTS IN THE ISOCRATIC RETENTION OF 50,000-MOL.WT. POLYSTYRENE ON THE 6-nm-PORE PACKING, WITH $\varphi = 0.87$

Temperature (°C)	*Flow-rate (ml/min)*	*Replicate values of k'*	*Av. k'*
23	0.5	3.85,3.56,3.65,3.60, 3.59	3.65 ± 0.11
	1.0	4.09,4.39	4.24 ± 0.21
	2.0	4.28,3.59	3.94 ± 0.26
45	0.5	0.67,0.72,0.62,0.61	0.66 ± 0.05
	1.0	0.68,0.58,0.58,0.59	0.61 ± 0.05
	2.0	0.65,0.65,0.66	0.65 ± 0.01

effectively achieved within the column during elution. In corresponding studies with normal-phase systems[20], as pronounced dependence of k' on flow-rate was observed for molecules as large as this.

The studies of ref. 20 also suggest that k' is strongly dependent on temperature in the case of larger polystyrene molecules. We have therefore carried out a few measurements on the temperature dependence of compounds in our system as a function of solute molecular weight. The data of Table XII suggest a temperature dependence of about -9% per °C for the 50,000-mol.wt. polystyrene, when $k' = 3.9$ (23°C). Similar data for the 800-mol.wt. polystyrene suggest a temperature dependence of k' equal to 1–2% for $2 < k' < 5$ (varying φ), again for the 6-nm-pore column (as in Table XII). A systematic study of temperature effects in reversed-phase systems has been reported[21] for typical small molecules, showing for similar k' values a change in k' of 1–2% per °C. Thus it appears that the temperature dependence of k' for the 50,000-mol.wt. polystyrene is about five-fold greater than would have been predicted on the basis of the behavior of molecules in the mol.wt. range 100–1000. This reflects a greater than expected entropy of retention for larger molecules, possibly as a result of unfolding from a highly compact (and organized) structure in the mobile phase prior to reversed-phase retention (see preceding section).

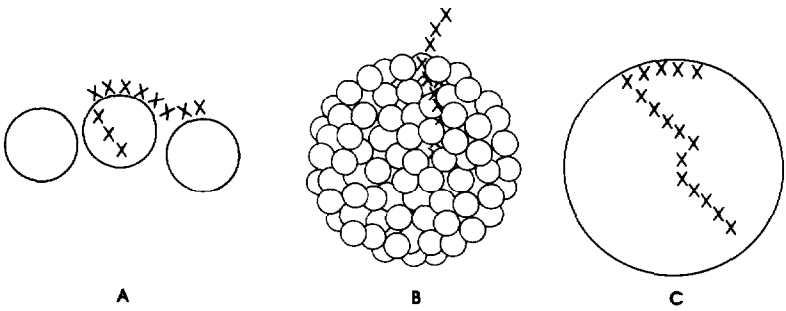

Fig. 8. Possible reasons for entropic exclusion of polystyrene molecules from stationary phase. A, *Bridging* of microparticles which form the pore structure of the column packing by a single polystyrene molecule (shown as × × × ×); B, *partial entry* of polystyrene molecule into pore of packing material; C, *confinement* of polystyrene molecule within a region of defined boundaries within the pore network.

Anomalous behavior of 50,000-mol.wt. polystyrene in Table IX

Values of V_s/V_{100} in Table IX for the 50,000-mol.wt. polystyrene are significantly lower than the average value $\bar{V}_s/V_{100}$ for lower-molecular-weight solutes. This is shown as the ratio $V_s/\bar{V}_s$ in Table IX, which indicates the reduction in V_s for the 50,000-mol.wt. solute. Thus, relative to the value of V_{100} for the latter sample, there is an apparent reduction in V_s for smaller-pore packings of 40% (30-nm pore), 69% (15-nm pore) and 72% (6-nm pore). This is also reflected in a greater deviation (± 0.2 units) in values of $\log(K_{0.6}V_{100})$ in Table X for the 50,000-mol.wt. polystyrene *vs.* ± 0.1 unit for lower-molecular-weight solutes. It is possible that a molecule as large as the 50,000-mol.wt. polystyrene suffers entropic exclusion within the stationary phase, for any of various reasons. Thus, Fig. 8 illustrates some of the configurations of a retained solute molecule (polystyrene) that might lead to a reduction in retention on narrower-pore packings. Further work is in progress with larger molecules to better define this effect, if it is real.

CONCLUSIONS

A number of conclusions and insights into the retention of large molecules in reversed-phase systems are possible on the basis of the present study:

(1) For the present packings and polystyrene solutes, it appears that the soft-ball model of Fig. 1 provides a much better description of the experimental data than is provided by the hardball model. Thus it appears that polystyrene molecules of any size ($200 \leqslant$ mol.wt. $\leqslant 50{,}000$) have access to essentially all of the stationary phase contained within the pores of both wide-pore (100-nm) and small-pore (6-nm) packings.

(2) The reversed-phase distribution constants, K_{1c}, for a given solute and particular mobile phase composition are constant for different column packings, implying that the stationary phase is equivalent so far as retention is concerned. However, the structure of the silica particles used to prepare these C_{18} bonded phases are essentially similar (Zorbax, Zipax), consisting of bonded microspheres of narrow size range.

(3) The effective volume of the stationary phase, V_s, inferred from these studies is proportional to the surface area of the starting silica packing, but with some fall off in V_s with narrower-pore packings (Fig. 5).

(4) Gradient elution can be used to conveniently determine isocratic retention parameters (k', K_{1c} V_s, S, etc.) for high-molecular-weight solutes. Values of these gradient-derived parameters are in reasonable agreement with corresponding isocratic data, although further study of causes for error in such measurements appears warranted. The resulting derived parameters permit the calculation of gradient retention, t_g values, with high precision (± 0.1 min in t_g, for $6 \leqslant t_g \leqslant 127$ min). This suggests that the present model and retention parameters can be used to optimize the separation of the polystyrenes with a high degree of reliability.

(5) The behavior of the higher-molecular-weight polystyrenes is similar in many respects to that of small molecules in similar reversed-phase systems. Thus, the dependence of k' on change in φ (S values) varies regularly from small (less retained) to large (strongly retained) solutes, with values of S being quite large for large polystyrene molecules. Similarly, the retention of these large molecules appears to rep-

resent an equilibrium process, as implied by constancy in k' as flow-rate and sample size are varied.

(6) In other respects, there are differences in the behavior of larger molecules in these reversed-phase systems. Thus, the dependence of k' on temperature increases for larger polystyrenes, and is about five-fold greater than expected (9% per °C *vs.* 1.5% per °C) for a 50,000-mol.wt. polystyrene *vs.* small molecules with similar k' values. Also, the Martin equation fails for polystyrenes with molecular weights greater than about 2000.

Efforts in this laboratory continue in an attempt to further define the value of gradient elution in studying and achieving the separation of macromolecular solutes.

APPENDIX I

Derivation of eqns. 2 and 3

If both sides of eqn. 1 are divided by the flow-rate, F, we obtain

$$t_R = t_{sec} + K_{1c}\,(V_s/V_m)t_0 \tag{A1}$$

where t_R is the band retention time (min), t_{sec} is the value of t_R with a strong mobile phase (*e.g.*, THF, see Table II) such that K_{1c} is zero, V_m is the total volume of mobile phase within the column (equal to V_0 plus V_i), and t_0 is the column dead-time (min). Note that $F = V_m/t_0$, $t_R = V_R/F$ and $t_{sec} = V_{sec}/F$. The capacity factor, k', for reversed-phase retention can be defined in the usual way:

k' = (amount of solute in stationary phase)/(amount of solute in mobile phase)

If the concentration of solute X in the mobile and stationary phases is $(X)_m$ and $(X)_s$, respectively, then

$$k' = (X)_s V_s/(X)_m V_{eff} \tag{A2}$$

where V_{eff} is the *effective* volume of the mobile phase. Since a partially excluded solute has a lower concentration in the volume V_i *vs.* the volume V_0, we can replace V_{eff} in eqn. A2 by $(V_0 + K_{sec}V_i)$, yielding:

$$\begin{aligned} k' &= (X)_s V_s/(X)_m(V_0 + K_{sec}V_i) \\ &= (X)_s V_s/(X)_m V_{sec} \end{aligned} \tag{A3}$$

Note that for total exclusion of the solute, $K_{sec} = 0$, and the effective mobile phase volume is V_i; for total permeation of solute, $K_{sec} = 1$, and exclusion effects can be ignored. Since $K_{1c} = (X)_s/(X)_m$, eqns. A1 and A3 then give eqn. 2.

Similarly, the above definition of K_{1c} allows eqn. A2 to be written

$$k' = K_{1c}V_s/V_{sec}$$

which then yields eqn. 3.

APPENDIX II

Derivation of eqn. 8 for retention time in gradient elution when size-exclusion effects are present and a lagtime exists between mobile phase mixing and the column inlet

The derivation of eqn. 7 (no SEC effects) is given in ref. 14 (p. 283). The starting point there is the eqn. A4:

$$\int_0^{V_g} (dV/V_a) = 1 \tag{A4}$$

Here, V_g is the corrected value of t_g (equal to $t_g - t_0$), V is the volume of mobile phase passing through the band center during its elution from the column, and V_a is the actual corrected retention volume at any time during elution; $V_a = K_{1c}V_s$. From eqn. 3 we have

$$k' = K_{1c}(V_s/V_{sec})$$

and

$$V_a = k'V_{sec} \tag{A5}$$

Eqn. 6 can be written (see ref. 14)

$$\log k' = \log k_0 - b(V/V_m) \tag{A6}$$

and the combination of eqns. A5 and A6 with insertion of the resulting expression for V_a into eqn. A4 gives:

$$\int_0^{V_g} 10^{bV/V_m}\, dV/V_{sec}\, k_0 = 1 \tag{A7}$$

Integration of eqn. A7 then yields:

$$V_g = (V_m/b) \log [2.3\, bk_0(V_{sec}/V_m) + 1] \tag{A8}$$

Division by F gives the corrected retention time ($t_g - t_0$), or:

$$t_g = (t_0/b) \log[2.3bk_0(t_{sec}/t_0) + 1] \tag{A9}$$

If the solute band does not migrate significantly in the starting mobile phase (k_0 large), then the lagtime, t_L, is simply added to the total retention time, t_g, yielding eqn. 8. Elsewhere we will discuss the case where k_0 is not large and there is a significant lagtime.

SYMBOLS

A, B, C	Constants in eqns. 5a, 5b
b	Gradient steepness parameter; eqns. 6, 6a
b_1, b_2	Values of b for two different gradient separations, where only the gradient time, t_G, is changed
b_5	Value of b for $t_G = 5$ min
C_{18}	Refers to dimethyloctadecylsilyl bonded phase
d_{18}	Apparent thickness of C_{18} bonded-phase layer; eqn. 4
F	Flow-rate (ml/min) of mobile phase
$(k_i)_j$	Value of k' for $\varphi = i$ and packing of pore diameter j (nm)
k'	Solute capacity factor, equal to amount of solute in stationary (bonded) phase divided by amount of solute in mobile phase; eqn. 2
k_i	Value of k' in gradient elution for mobile phase entering column at some time t; eqn. 6
k_0	Value of k_i at time zero, assuming lagtime, t_L, equal zero
k_w	Value of k' for water as mobile phase; eqn. 5
K	Solute distribution constant for either reversed-phase or SEC separation
K_i	Value of K for reversed-phase retention with $\varphi = i$; $K_{0.6}$ is the value of K for 60% (v/v) THF–water
$(K_i)_j$, $(K_i)_k$	Value of K_i for column packing of pore diameter (nm) j or k; $(K_{0.6})_{15}$ is value of K for 60% (v/v) THF–water and 15-nm-pore packing
$(K_j/K_k)_{sec}$	K_j and K_k refer to SEC K values for column packings of pore diameter (nm) j and k; $(K_{100}/K)_{sec}$ refers to ratio of K values for 100-nm-pore packing and some other packing
K_{1c}	Value of K for reversed-phase retention
K_{sec}	Value of K for SEC retention
$K_{0.6}$	Value of K_i for $\varphi = 0.6$
n	Number of styrene repeat units in polystyrene molecule; eqn. 5b
S	Slope of plot of log k' *vs.* φ; eqn. 5
$(SA)_j$	Surface area of silica of pore diameter (nm) j; $(SA)_{100}$ is surface area of silica of pore diameter 100 nm
t	Time (min) after sample injection or start of gradient; eqn. 6
t_g	Retention time (min) of solute in gradient elution
t_{g1}, t_{g2}	Values of t_g for two gradient runs where only t_G is varied (t_G equal t_{G1} and t_{G2}, respectively)
t_G	Gradient time (min); elapsed time from beginning of gradient to end
t_{G1}, t_{G2}	Values of t_G for two different gradient runs, with different resulting values of b (b_1, b_2) and t_g (t_{g1}, t_{g2})
t_L	Lagtime (min) for gradient system; equal to time required for mobile phase to move from gradient mixer to column inlet
t_0	Column dead-time (min); time required for mobile phase molecules to traverse column
t_R	Retention time (min) of solute band
t_{sec}	Retention time, t_R, for SEC retention only ($K_{1c} = 0$)
V_i	Volume (ml) of mobile phase inside pores of column packing

V_j, V_k	Value of V_s for column packing of pore diameter (nm) j or k
V_m	Total volume (ml) of mobile phase inside column (equal to $V_i + V_0$)
V_0	Volume (ml) of mobile phase inside column but outside pores of packing
V_R	Retention volume (ml) of solute band
V_s	Effective volume of stationary phase inside column (nominally, the volume of C_{18} bonded phase)
V_{sec}	Value of V_R for SEC retention only ($K_{1c} = 0$)
$\bar{V}_s$	Value of V_s for given column packing and various polystyrenes with mol.wt. less than 50,000; $V_s/\bar{V}_s$ for 50,000-mol.wt. polystyrene in Table IX is then the apparent fraction of $\bar{V}_s$ available to the 50,000-mol.wt. solute ($\bar{V}_s$ is the average V_s for smaller molecules)
V_{100}	Value of V_s for 100-nm-pore packing
$(X)_m$, $(X)_s$	Concentrations of solute X in mobile and stationary phases, respectively
$\Delta\varphi$	Change in φ during gradient run; a gradient from 60 to 100% (v/v) THF has $\Delta\varphi = 0.40$
φ	Volume fraction of organic solvent (THF) in organic–water mobile phase

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