CHROM. 18 464

GENERAL MODEL FOR THE SEPARATION OF LARGE MOLECULES BY GRADIENT ELUTION

SORPTION *VERSUS* PRECIPITATION

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(First received November 21st, 1985; revised manuscript received January 6th, 1986)

SUMMARY

Solute retention in the gradient elution separation of polymers and other macromolecules can occur by either sorption of individual molecules to the stationary phase or by a precipitation–redissolution process. Which process applies in a given situation depends on the sample solubility, the strength of the interaction of sample molecules with the stationary phase and the amount of sample injected on to the column. A general model is presented for these separations, and experimental data for the reversed-phase gradient elution separation of a 50 000-dalton polystyrene sample are reported and compared with the model. In this case, "normal" retention by a sorption process occurs for samples smaller than 200 μg.

INTRODUCTION

Following the introduction of high-performance liquid chromatography (HPLC) in the late 1960s, much of the next decade was devoted to exploiting this unique separation method for the purification and analysis of mixtures of small molecules (which we define as having molecular weights of less than 10 000 daltons). After almost two decades of "small-molecule" HPLC, we now have a good quantitative basis for understanding and controlling these separations[1-4]. There is decreasing disagreement among experts over such fundamental issues as band spreading, retention processes ("mechanisms"), mobile phase effects and special techniques (*e.g.*, gradient elution). Our knowledge has reached the point where in many instances it is possible to predict quantitatively what will happen to retention, band width and resolution as we change separation conditions[5-7].

During the past 5 years a similar revolution has occurred in our ability to separate large molecules by various HPLC procedures. The application of this research has been mainly in the life science area[8-12], but synthetic polymer chemists have also benefited[13]. Despite the great promise of HPLC for the separation of these macromolecule samples, our understanding of these separations lags behind that for the chromatography of small molecules. There is also a concomitant lack of agreement among many workers as to the processes involved in large-molecule separations, and how these differ from those for small molecules. There is no question that in some respects large molecules have unique chromatographic properties. However, it does not necessarily follow that the basic separation process for these samples differs essentially from that which describes corresponding small-molecule separations.

Most separations of individual macromolecules are today carried out by gradient elution. Many workers have commented on the "strange" behavior of large-molecule gradient elution[11,14-21], and some chromatographers have suggested that new models of the separation process are required to explain these observations. In many instances, however, the reported "anomalies" appear to represent a misunderstanding of what to expect in such cases (see, *e.g.*, the discussion in ref. 22). Gradient separations of large molecules are often compared directly with corresponding small-molecule isocratic separations. Although the same chromatographic process generally holds for each of these two situations, the observed results will have predictable differences. In other instances it is clear that effects ordinarily excluded from small-molecule theory must be considered when large molecules are involved, especially biological macromolecules.

Among the alternatives to conventional chromatographic retention for large solute molecules, four possibilities have received frequent comment: (a) the "pop-off" proposal, which assumes that a solute is desorbed from the column inlet at some time during the gradient, and does not re-attach to the stationary phase during its subsequent migration through the column[11,19,20]; (b) multi-site retention, which assumes multiple attachments between the solute molecule and the stationary phase[14]; (c) "critical solution behavior"[16,23-28]; and (d) precipitation–redissolution as described most recently by Glöckner and co-workers[15,29-33].

We have shown[22] that the first two cases ("pop-off" and "multi-site" retention) are actually special cases (*i.e.*, logical extensions) of normal chromatography, and therefore do not require further comment. "Critical solution behavior" is considered in the following paper[34], where we show that arguments on its behalf are either ambiguous or in error. In this paper we shall see that normal chromatographic retention and precipitation–redissolution represent two extreme processes, either of which can determine chromatographic separation in a given case. Here we develop a general model that clarifies certain points of previous controversy.

THEORY

Precipitation–redissolution model

The precipitation–redissolution model describes the HPLC separation of polymer samples. We shall therefore discuss this special case, although our conclusions should also be applicable to other macromolecule samples.

The use of precipitation–redissolution for the fractionation of synthetic poly-

mers on column beds is not new. The first examples appeared in 1950 when Desreux and Spiegel[35] eluted polymers that had been precipitated on to a sand-packed column with a series of solvent–non-solvent mixtures of increasing solvent power. Essentially a single-step extraction analysis, the rate and efficiency of redissolution were usually complicated by occlusion and coprecipitation, and also crystallinity in the initial precipitate (for further discussion, see ref. 36).

The use of a temperature gradient in addition to the solvent gradient was later introduced by Baker and Williams[37]. In subsequent development of a theory for Baker–Williams fractionation[38–40], the term "precipitation chromatography" was adopted, as the polymer was extracted by the solvent gradient, and then reprecipitated as it eluted into a cooler zone in the column. Inefficiencies arising from nonideal morphology of the precipitate were reduced in multiple (continuous) fractionations provided by the combined gradient system. The reader is referred to the review by Barrall and Johnson[41] for further details.

Inagaki[42] presented the first examples of thin-layer precipitation chromatography on modern, porous chromatographic adsorbents in 1971. Numerous examples of this technique followed (for synthetic polymers, see refs. 42–45). Glöckner and co-workers[15,29–33] more recently described how precipitation chromatography on porous media is a multi-stage process akin to Baker–Williams fractionation. Instead of a thermal gradient, the exclusion of polymers from small pores is used to precipitate the sample continuously. In the Glöckner model it is assumed that (a) smallpore packings are used that can be permeated by solvent molecules, but not by solute molecules, and (b) retention is controlled by solute solubility in the mobile phase (solute molecules leave the mobile phase by "precipitating" onto the surface of the column packing). If the volume of mobile phase outside the packing pores is V_e and the volume inside is V_i, then polymer (solute) molecules (in the solubilized state) will move through the column with a velocity u_p equal to

$$u_p = LF/V_e \tag{1}$$

where L is the column length and F is the mobile phase flow-rate. Similarly, the velocity of solvent molecules moving through the column will be

$$u_s = LF/(V_e + V_i) \tag{2}$$

Therefore, solute molecules in solution move along the column more rapidly than do solvent molecules, and the sample tends to move from a stronger ("better") solvent into a weaker ("poorer") solvent because of the mobile phase gradient. When this occurs, the solute precipitates, until it is overtaken by stronger ("better") solvent and redissolves. This process occurs many times during the migration of sample through the column, leading to fractionation of the sample. Alternatively, if the polymer molecules permeate the pores of the column packing, a sample band will move down the column when solvent of the right composition arrives at the column inlet. This latter process would presumably be less effective at separating sample components than where exclusion of polymer occurs.

Ideally, precipitation of the polymer is independent of the nature of the column packing material, i.e., precipitation is determined solely by (solvent–non-solvent)–polymer phase equilibria. However, the most recent version of the Glöckner model

also accepts the (alternative) possibility of direct interaction of isolated ("precipitated") polymer molecules with the surface of the column packing. While most chemists assume that precipitation involves the formation of a solid phase of the substance being precipitated (in which case the free energy of this phase is essentially independent of the surroundings of the precipitate), Glöckner and Van den Berg[32] refer to the importance of adsorption interactions between precipitated solute molecules and the column packing surface, as have numerous earlier users of Baker–Williams and column elution methods (see discussion of in ref. 41).

This apparent confusion is addressed in this paper. As we shall see, two retention possibilities exist that can be described variously as (a) precipitation or (b) sorption or normal retention, the latter involving interaction of isolated polymer molecules with the surface of the column packing.

Normal chromatographic retention model ("sorption")

Let us pursue the consequences of the two possible retention processes we have just defined: (a) precipitation–redissolution and (b) adsorption (or sorption) of isolated sample molecules. We begin by considering a sample that consists of a single polymer molecule (typically of molecular weight $< 10^7$ daltons), injected onto an HPLC column and followed by gradient elution. Here there is no question of precipitation in the classical sense, so that what we define as normal chromatographic retention is expected. Normal behavior would also be expected for larger samples of a polymer sample that is relatively soluble in the particular mobile phase employed.

The resulting chromatogram for the latter case ("normal" retention) is shown in Fig. 1. The composition of the gradient (at the column outlet) is shown as the dashed curve, which begins with a composition φ_0 (φ is the volume fraction of strong solvent in the mobile phase) and ends with a composition φ_f. Note that the mobile phase composition (column outlet) does not change until a time $t_0 + t_D$ after the start of the gradient, where t_0 is the column dead time and t_D is the gradient delay time of the HPLC system (the time required for a change in mobile phase composition in the gradient mixer to reach the column inlet). The center of the sample band is seen to elute at some retention time t_g; the mobile phase composition at elution of the band is defined as φ_e. The gradient is completed at time $t_G + t_0 + t_D$, where t_G
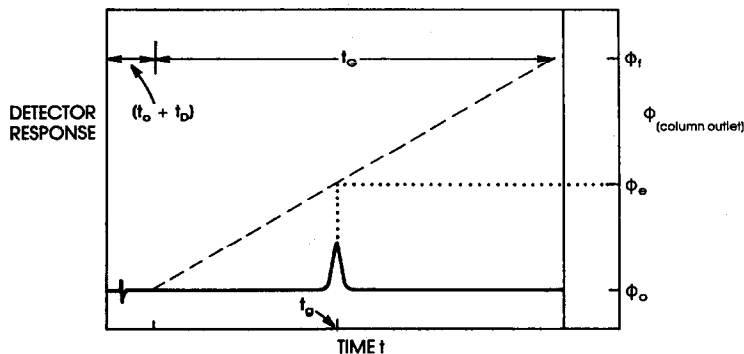

Fig. 1. Schematic representation of polymer separation by gradient elution.

is the gradient time. We assume in the following discussion that the band in question elutes at a time between $t_0 + t_D$ and $t_G + t_0 + t_D$, *i.e.*, during the gradient.

Several useful retention relationships can be derived for the example in Fig. 1 (*cf.*, refs. 46–50, gradient elution theory for normal chromatographic retention). We shall assume linear solvent strength (LSS) behavior[46] and a reversed-phase HPLC system. The solute capacity factor $\bar{k}$ can be defined for the band at the time it is at the column midpoint; $\bar{k}$ is given by

$$\bar{k} = 0.87 t_G F/(V_m \Delta\varphi S) \qquad (3)$$

where F is the mobile phase flow-rate, V_m is the column dead volume (equal to t_0), $\Delta\varphi$ is the change in φ during the gradient (equal to $\varphi_f - \varphi_0$) and S is the value of $-d(\log k')/d\varphi$ for the solute in a corresponding isocratic HPLC system.

The retention time t_g (for "gradient" conditions: $\varphi_e \gg \varphi_0$) is

$$t_g = (t_0/b)\log (2.3 k_0 b) + t_0 + t_D \qquad (4)$$

where

$$b = 1/1.15\bar{k} \qquad (5)$$

and k_0 is the value of k' for $\varphi = \varphi_0$. Eqns. 4 and 5 then yield

$$t_g = (1.15 t_0 \bar{k})\log(2 k_0/\bar{k}) + t_0 + t_D \qquad (6)$$

The quantity φ_e is given by

$$\varphi_e = [(t_g - t_0 - t_D)/t_G] \Delta\varphi + \varphi_0 \qquad (7)$$

which with eqns. 3 and 6 can be expressed as

$$\varphi_e = (1/S)\log(2 k_0) - (1/S)\log(\bar{k}) + \varphi_0 \qquad (8)$$

For tetrahydrofuran–water mobile phases, it has been shown[34,49,50] that for polystyrene solutes S is related to the solute molecular weight M by

$$S = 0.22 M^{1/2} \qquad (9)$$

Mixed retention mode model

There is no *a priori* reason to assume that either the normal retention or precipitation–redissolution model will describe all gradient elution polymer separations. A very small sample of a relatively soluble polymer will be more likely to undergo normal retention, whereas a large injected sample of a less soluble polymer should separate by the precipitation–redissolution process. The essential features of this situation are shown in Fig. 2a. The ordinate corresponds to values of φ (solubility studies) or φ_e (gradient separation) and the abscissa corresponds to values of log C_{max}; C_{max} is the (saturated) mobile phase concentration of the polymer in the solubility studies (also called C_s), or the concentration of the band maximum in gradient elution (at the time of elution). The quantity C_{max} is also approximately proportional to the total weight, w_x, of polymer injected onto the column.
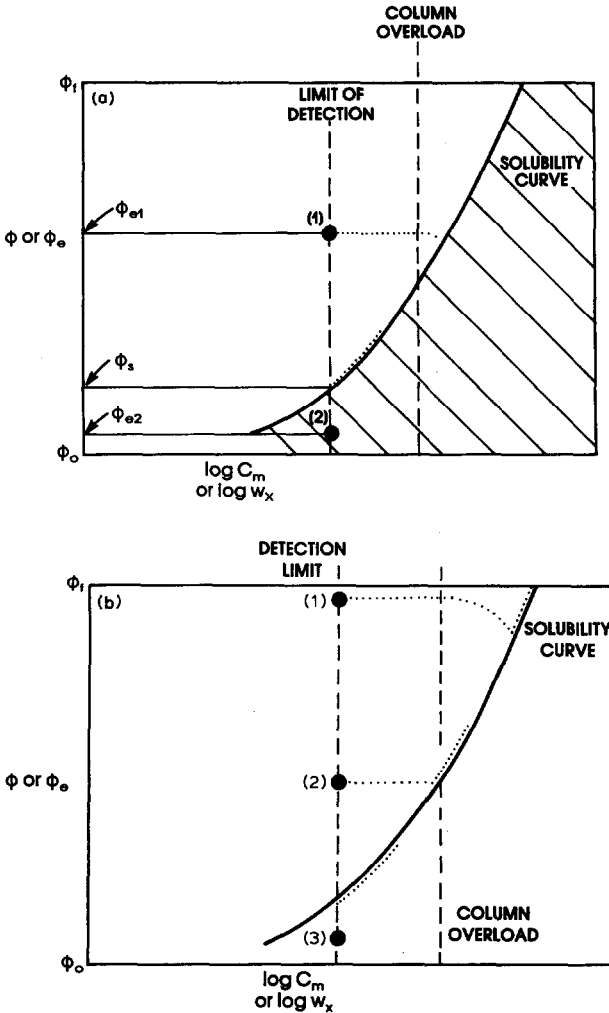
Fig. 2. Visualization of "normal" and "precipitation" retention processes. Mobile phase composition at elution (φ_e) *vs.* sample size. (a) Normal retention of sample 1 and precipitation–retention of sample 2 for just-detectable sample sizes; (b) mixed retention observed for wide range in sample size (1, normal retention, overload and precipitation observed; 2, normal retention and precipitation observed; 3, precipitation only observed). (····), Dependence of φ_e on w_x.

Normal retention. Two examples are shown in Fig. 2a. Example 1 is a polymer sample that elutes in a mobile phase composition φ_{e1} under normal retention conditions (*e.g.*, injection of a single molecule of polymer). This sample is also eluted at the same value of φ (φ_{e1}) for a sample size just large enough to be detected with a given set of conditions (choice of detector, etc.); this run is indicated by the solid circle in Fig. 2a labeled 1. The solubility curve (heavy curve, φ *vs.* C_{max}) is also indicated in Fig. 2a for this sample. Note that for the minimum detectable sample size (value of C_{max}), normal retention of the sample dominates, because for this sample the band elutes at a concentration C_{max} that is well below the solubility limit

for the mobile phase composition φ_{e1}. Or we can say that normal retention is so strong that the value of φ_e is greater than the value of φ that would just precipitate this mass of sample (*i.e.*, where C_{max} corresponds to a saturated solution of composition φ).

At the beginning of the gradient ($\varphi = \varphi_0$), it is seen that the sample corresponding to data point 1 is insoluble in the mobile phase. At some later time (when $\varphi = \varphi_s$) the sample dissolves, but φ is not large enough to elute the sample (k' is very large at this point in the separation). That is, the initially precipitated sample becomes strongly attached to the stationary phase, and polymer–polymer interactions are replaced by polymer–stationary phase interactions.

The dotted line extending to the right of point 1 in Fig. 2a corresponds to the dependence of φ_e on C_{max} for this sample as the sample size is increased. For this particular case, φ_e is initially not a function of sample size, because (i) the solubility limit of the sample band has not yet been reached, and (ii) the surface of the stationary phase has not yet been overloaded with sample. The column overload limit is indicated in Fig. 2a as a vertical dashed line, and until this sample size is attained, φ remains constant with increase in sample size. For sample sizes larger than the column overload value, retention decreases as seen in Fig. 2a. Finally, for sufficiently large samples, the plot of φ_e *vs.* C_{max} intersects the solubility curve, at which point the sample precipitates, and the precipitation–redissolution retention process takes over.

Retention by precipitation. Data for a second polymer sample (2) are also shown in Fig. 2a. For convenience, it is assumed that the detection limit and solubility curve for this sample are the same as for the polymer sample in the preceding example (1). However, polymer 2 is much less strongly retained by the stationary phase, so that its φ_e value (very small sample) is smaller than in the preceding case. For sufficiently small samples, polymer 2 would elute in a mobile phase composition φ_{e2}. However, for a sample large enough to be detected, polymer 2 is precipitated in mobile phase of composition φ_{e2}. In this case the elution of minimum detectable samples of polymer 2 will occur at $\varphi_e = \varphi_s$, the solubility limit of the polymer. With further increase in sample size (dotted line in Fig. 2a), polymer 2 will show φ_e increasing with increase in sample size. This is observed to be the opposite of the behavior of polymer 1 (φ_e is constant or decreasing with increase in w_x).

Mixed retention mode. If the sample size is varied over wide enough limits, and if very low concentrations of eluted polymer can be detected, most samples should exhibit both normal and precipitation–redissolution retention behavior. This is further illustrated in the similar examples in Fig. 2b. However, in most preceding studies of polymer gradient elution, variation in sample size has been too limited to demonstrate both retention modes for a given polymer sample, relative to the range in sample size normally required.

Isocratic retention

Normal retention. Various workers have commented on their apparently inability to elute high-molecular-weight polymer samples isocratically[51]. Although it is true that such samples can be eluted (with $k' > 0$) only within a very narrow range of mobile phase compositions (narrow range of φ values), we have observed clear isocratic elution (tetrahydrofuran–water mobile phases) of a 50 000-dalton polysty-

rene at φ values from 0.855 ($k' = 7$) to 0.90 ($k' = 0.1$) (15 nm pores, C_{18} column[49,50]. Further, these isocratic values of k' *vs.* φ are accurately predictable from retention data obtained by gradient elution for the same HPLC system, using retention theory for normal retention[49]. The latter system therefore does not differ essentially in the retention process ("normal" behavior) from the case of small-molecule separations.

Precipitation retention. Precipitated polymer will be contacted continuously by fresh solvent during isocratic elution. For the simple case of a single solvent–high-molecular-weight polymer combination, the critical volume fraction, v_{2c}, of polymer at which precipitation will occur (theta condition) is

$$v_{2c} = (1 + x)^{-1/2} \tag{10}$$

where x is the number of polymer repeat units[52]. In the isocratic elution of a precipitated sample, a large volume of solvent will often be needed to extract (elute) an injected sample of significant mass (*e.g.*, 1–100 μg). The solvent volume will vary with v_{2c} and therefore with sample molecular weight (or value of x).

The dependence of the resulting elution curve as a function of polymer solubility and sample size is shown in Fig. 3 for monodisperse polymer samples. In each instance, elution begins at a time t_{sec}, where $t_{sec} = V_{sec}/F$, and V_{sec} is the volume or space within the column accessible to the polymer molecule (see ref. 50). For polydisperse samples, a similar elution pattern will be observed, except that severe tailing of the sample elution band would occur (Fig. 3c). The appearance of incomplete elution or split peaks can be observed for samples that are highly polydisperse, because of the dependence of theta conditions on x:

$$\chi_{1c} = (1 + x^{1/2})^2/2x$$
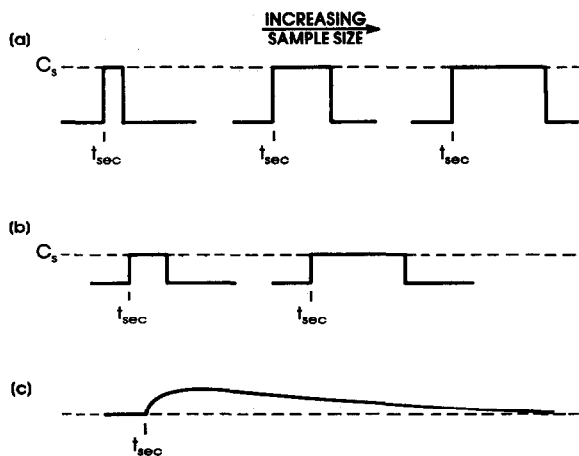$$\approx 1/2 + x^{-1/2} \tag{11}$$

Fig. 3. Expected isocratic elution bands for a system where precipitation–redissolution governs retention. (a) Monodisperse polymer sample, higher concentration of "good" solvent; (b) same, lower concentration of "good" solvent; (c) polydisperse polymer sample.

where the interaction parameter χ_{1c} is proportional to the volume fraction of good (or "strong") solvent needed for elution.

In the isocratic elution of either mono- or polydisperse samples it will be impossible to obtain Gaussian (or any shape) elution bands with retention times greater than t_{sec} as long as the precipitation retention mode prevails. Thus the isocratic elution of a polymer sample to yield a near-Gaussian band with retention time $t_R > t_{sec}$ is compelling evidence that normal retention prevails in that case.

Gradient retention and sample solubility

Previous workers[30] have noted that φ_e values for polymers of varying molecular weight often correlate with polymer solubility of the "cloud point" of the sample. This has frequently been considered as evidence of a precipitation retention process. In reversed-phase separations of small molecules, however, the same dependence of retention on sample solubility is often observed[53,54], despite the complete solubility of the sample under chromatographic conditions. This can be rationalized in terms of strong interactions between solvent molecules and/or between sample and solvent molecules in the mobile phase, with only weak interactions between the stationary phase and these same molecules (see discussion in refs. 27 and 53).

EXPERIMENTAL

The HPLC system was a DuPont 8800 liquid chromatograph (DuPont, Wilmington, DE, U.S.A.) equipped with a Model 860 fixed-wavelength (254 nm) photometric detector and heated column compartment. All runs were carried out at 35°C, using 15 nm pore C_{18} column packings (DuPont Zorbax C18-150) and linear gradients from 75% to 95% (v/v) tetrahydrofuran (THF)–water unless stated otherwise. The sample was a 50 000-dalton monodisperse polystyrene (Pressure Chemicals, Pittsburgh, PA, U.S.A.). The conditions and procedures were similar to those described in refs. 46, 48 and 50.

RESULTS AND DISCUSSION

We have previously published data on the gradient elution separation of polystyrenes of varying molecular weight (800–233 000 daltons) using THF–water as mobile phase and C_{18} reversed-phase columns of varying pore size[49,50]. We described these separations as examples of normal chromatographic retention, as opposed to precipitation–redissolution. This interpretation has been questioned[16], although further analysis of this system[50] shows excellent agreement between experimental data and predictions of a model based on normal chromatographic retention. Because of the considerable amount of data already reported by us for this particular system, we have examined it further in terms of normal retention *vs.* precipitation–redissolution.

Solubility data

The discussion of Fig. 2 in the Theory section makes it clear that the solubility of the polymer sample as a function of mobile phase composition φ is of critical importance in interpreting the relative importance of normal *vs.* precipitation reten-

TABLE I

SOLUBILITY OF 50 000-DALTON POLYSTYRENE IN THF–WATER MIXTURES AT 35°C

THF concentration (%, v/v)	Sample solubility (g/l)
84	0.0072
85	0.0558
86	0.280
87	1.11

tion modes in gradient elution separations. We determined the solubility of the present 50 000-dalton monodisperse polystyrene sample in THF–water mixtures for various values of φ. The sample was first dissolved completely in pure THF, then an aliquot was added to a particular THF–water mixture to provide some known final value of φ. The sample was shaken at 35°C for 24 h and an aliquot was sampled for analysis of polymer content (by gradient elution HPLC). This procedure was shown to give reproducible and constant values of polymer solubility, independent of the elapsed time of contacting sample and solvent.

The resulting solubility data are summarized in Table I and plotted in Fig. 4 (heavy dashed curve). It can be seen that the solubility increases rapidly with increase in the "good" solvent (THF), as is typical of high-molecular-weight polymer samples. We note in passing that the data in Table I are not absolute values, as polymer solubility depends (slightly) on the ratio of polymer and solvent in the original mixture[52].

Gradient retention data versus amount of sample injected

Various amounts of the 50 000-dalton polystyrene sample were next injected under standard gradient elution conditions and retention times were measured. These
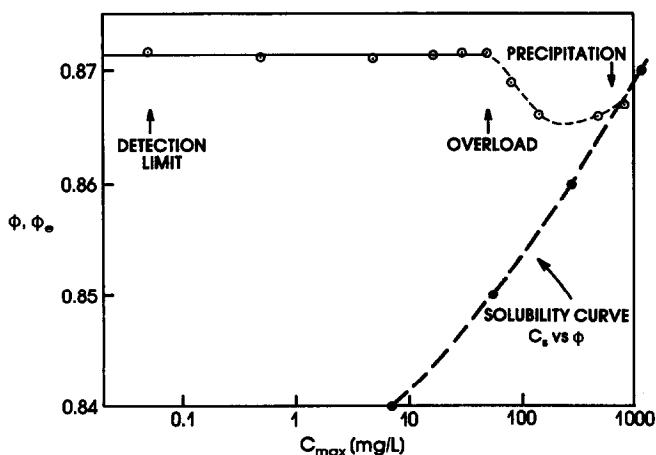

Fig. 4. Retention data for 50 000-dalton polystyrene sample. Gradient elution from C_{18} column by THF–water gradient; conditions as in Table II. Solubility curve from Table I.

TABLE II

RETENTION TIMES FOR 50 000-DALTON POLYSTYRENE SAMPLE AS A FUNCTION OF AMOUNT OF SAMPLE INJECTED

Conditions: 75–95% (v/v) THF–water gradient as described under Experimental; t_G = 30 min; F = 2 ml/min; t_0 = 1.41 min; t_D = 2.74 min; 15 μl sample volume (in THF); 35°C.

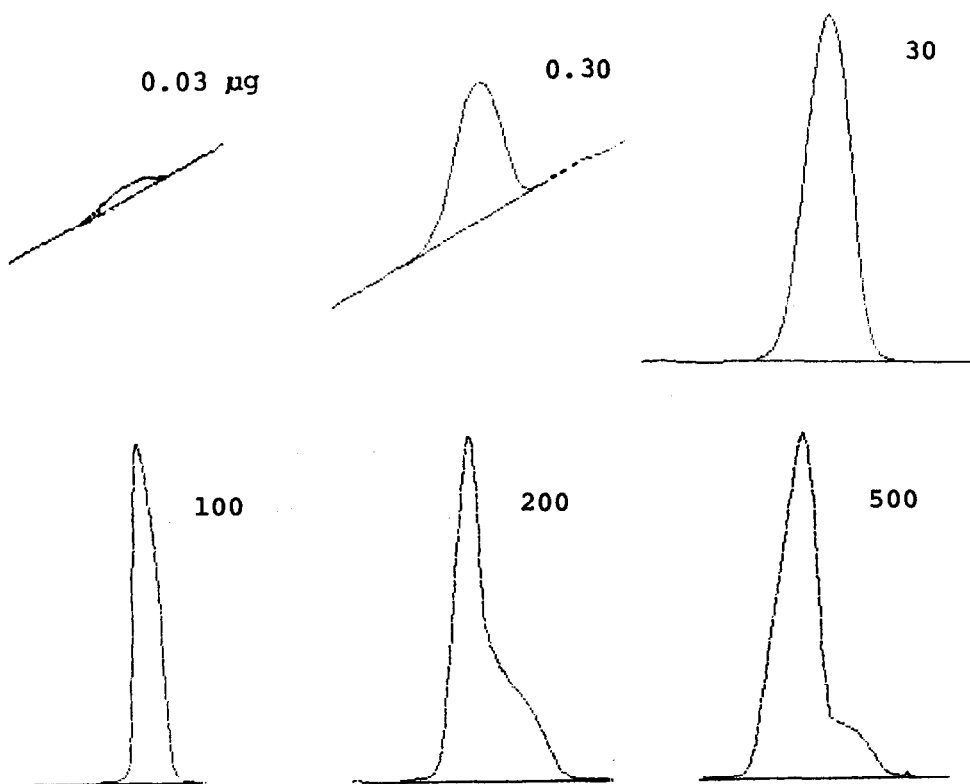| Weight of sample (μg)	t_g (min)	φ_e	$C_{max}{}^*$ (ml/l)
0.030	21.80	0.8717	0.050
0.30	21.77	0.8712	0.50
3.0	21.76	0.8711	5.0
10	21.80	0.8714	17
30	21.83	0.8716	50
50*	21.44	0.8690	84
100*	21.00	0.8661	142
200*	21.0	0.866	480
500*	21.1	0.867	830

* Larger sample volumes: 50–100 μl, 10 mg/ml.

Fig. 5. Band shape vs. sample size for gradient runs of Fig. 4; 50 000-dalton sample, THF–water gradients.

data are summarized in Table II, together with calculated values of φ_e and C_{max} (concentration at band maximum). Fig. 4 shows the dependence of φ_e on C_{max} for the data in Table II.

The plot in Fig. 4 closely resembles that for sample (a) in the theoretical plots in Fig. 2b. In this instance the detection limit for the system in Fig. 4 corresponds to a C_{max} value of about 0.05 mg/l (see the chromatograms in Fig. 5), and the overload limit occurs at about $C_{max} = 50$ mg/l. Over this range of C_{max} values (1000-fold), values of φ_e are constant within experimental error (for triplicate determinations of t_g, 1 standard deviation $= \pm 0.0003$ in φ_e). When $C_{max} > 50$ mg/l, the column begins to overload and values of φ_e decrease. As the sample size is increased further, the curve for φ_e vs. C_{max} approaches the solubility curve, and for C_{max} values of about 500 mg/l a precipitation retention mode becomes dominant. In the region of $C_{max} = 50$–500 mg/l, the band shape begins to change as shown in Fig. 5. The roughly Gaussian bands observed for small samples (0.03–30 μg) begin to distort for the 100 μg sample, with the development of a distinct "hump" for larger samples (200–500 μg).

Dependence of gradient retention data on sample solubility

The preceding discussion plus Fig. 4 makes it clear that small samples of the 50 000-dalton polystyrene are separated by a normal retention process. We have reported data on the dependence of k' on φ for this sample (isocratic elution[49]), and it is interesting to compare those data with the solubility of this sample vs. φ. Data on small-molecule systems suggest that k' should correlate inversely with solubility C_s, and this relationship is tested for the present sample in Fig. 6. Here values of k' are plotted vs. φ (squares), and the quantity $0.9/C_s^{\star}$ (l/g, circles) is superimposed on this plot. An excellent correlation is noted, with retention increasing as solubility decreases. This means that values of φ_e would also correlate closely with solubility or cloud-point measurements. However the interpretation of such a correlation as support for a precipitation mode of retention is not justified, as the data in Figs. 4 and 6 make clear. Only when φ_e values correlate exactly with C_s values (as for sample 2 in Fig. 2a) is such an observation capable of distinguishing between normal and precipitation retention modes.

Dependence of normal sample retention on experimental conditions

It is often noted that values of φ_e in the separation of both synthetic polymers and biological macromolecules do not change much with the experimental conditions: flow-rate F, gradient time t_G, column length L or the composition of the column packing. This has frequently been cited as evidence for a precipitation retention process, as the latter retention mode would indeed be unaffected by these experimental variables. However, this is also often true for normal retention. This can be seen by examining eqns. 1, 6 and 7 derived in the Theory section. First, eqn. 9 predicts that values of the solute parameter S increase markedly with increasing sample molecular weight, and S is often larger than 100[★★]. Eqn. 8 relates φ_e to values of $\bar{k}$, which are

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     * The quantity 0.9 is a best-fit value to the experimental data in Fig. 6, assuming an inverse relationship between solubility and retention.
     ** Large values of S for samples with large M are predicted for normal chromatographic retention on the basis of either a displacement process or mobile phase interactions[22].
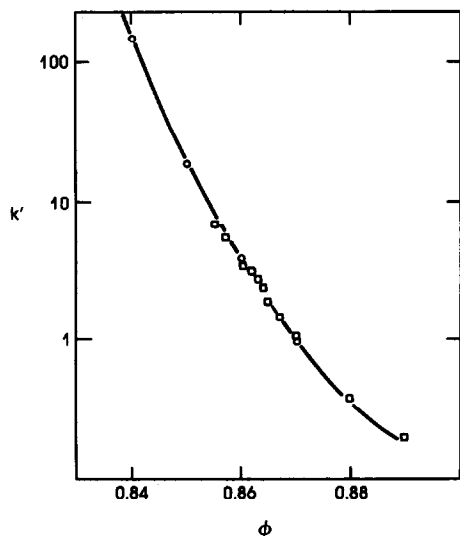
Fig. 6. Correlation of isocratic retention data (k' *vs.* mobile phase composition φ with inverse sample solubility ($0.9/C_s$). Data from Table I and ref. 49. $\bigcirc$, $0.9/C_s$ (l/g); $\square$, k' for isocratic elution.

affected by the experimental conditions as described by eqn. 1. We can combine eqns. 3 and 8 to obtain

$$\varphi_e = (1/S)\log(2k_0) - (1/S)\log[0.87t_GF/(V_m\Delta\varphi S)] + \varphi_0 \qquad (12)$$
$$\qquad\quad \text{(i)} \qquad\qquad\qquad \text{(ii)} \qquad\qquad\qquad \text{(iii)}$$

For large molecules, k_0 is generally very large, and terms i and iii in eqn. 12 largely determine the value of φ_e. In reversed-phase systems, we have also seen that the k' values are determined mainly by interactions of sample and solvent in the mobile phase, so that k_0 tends to be less dependent on column type. Values of k_0 should be proportional to the surface area of the column[34], but for an S value of 100 a doubling of the column surface area then increases φ_e by only 0.01(log 2) or 0.003 unit.

A similar doubling of gradient time, flow-rate or column length (or volume V_m) also changes φ_e by 0.3/S, *i.e.*, by very little when S is large. This suggests that the use of these experimental variables (column type, t_G, F, V_m, etc.) as a means of determining which retention process prevails in a given system requires very careful measurement of values of φ_e (typically ±0.002 in φ) as the experimental conditions are changed.

CONCLUSIONS

The separation of synthetic polymers by gradient elution can occur either by normal chromatographic retention or by a precipitation–redissolution process. Normal retention is favored with samples that are (1) more soluble, (2) more strongly retained by the stationary phase and (3) injected in smaller amounts. The retention process that actually occurs in a given system can be determined by measuring re-

tention as a function of sample size and comparing these data with the solubility of the sample as a function of mobile phase composition φ.

We have examined the reversed-phase gradient elution separation of a 50 000-dalton monodisperse polystyrene sample as a function of sample size (0.03–500 μg injected), and compared this with the solubility of this sample in the mobile phase (THF–water mixtures of varying φ). These data clearly show that for samples smaller than 200 μg a normal retention process occurs. For samples of 200 μg or larger, precipitation–redissolution becomes important. Previous data that we have reported on the similar separation of polystyrenes of varying molecular weight (refs. 49 and 50, small samples) appear to be governed by normal chromatographic retention as opposed to precipitation–redissolution. Other systems will have to be evaluated on a case-by-case basis to determine the nature of separation for each example.

ACKNOWLEDGEMENTS

GLOSSARY OF SYMBOLS USED IN THIS AND THE FOLLOWING PAPER[34]

A	a (negative) constant for polymers with molecular weights > 10 000 daltons; ref. 34, eqn. 8;
b	gradient parameter for linear-solvent-strength gradient elution; eqn. 5;
C_{max}	concentration of solute at band maximum when band elutes;
C_s	solute concentration in saturated solution for a given value of φ;
F	mobile phase flow-rate (ml/s);
k'	solute capacity factor;
$\bar{k}$	average or effective solute capacity factor in gradient elution; eqn. 3;
k_0	value of k' for mobile phase at beginning of gradient ($\varphi = \varphi_0$);
k_w	k' value for water as mobile phase (reversed-phase);
k_1, k_{-1}	forward and reverse rate constants for ref. 34, eqn. 3;
L	column length (cm);
LSS	linear solvent strength;
M	solute molecular weight (daltons); also degree of polymerization, proportional to mol.wt.;
S	change in k' as a function of mobile phase composition; equal to $-d(\log k')/d\varphi$;
t_D	dwell time of HPLC system (s); time required for change in mobile phase composition to move from gradient mixer to column inlet;
t_g	retention time in gradient elution (s); eqn. 4;
t_G	gradient time (s);
t_0	column dead time (s);
THF	tetrahydrofuran;
u_p	velocity of polymer band moving through column (cm/s);
u_s	velocity of solvent molecules moving through column (cm/s);
V_e	volume of mobile phase in column, but outside of pores (ml);
V_i	volume of mobile phase inside pores of column packing (ml);

V_m — volume of mobile phase inside column, equal to $V_e + V_i$ (ml);

v_{2c} — volume fraction of polymer;

w_x — weight of polymer injected onto column;

x — number of polymer repeat units;

X_m, X_s — a molecule X in the mobile (m) or stationary (s) phase;

X_m^*, X_s^* — a molecule X^* with conformation different from that of X (X and X^* are the same compound); ref. 34, eqn. 2;

$X_s^\#$ — a molecule X that is retained by a different process on the stationary phase (ref. 34, eqn. 3);

$\Delta \varphi$ — change in φ during gradient elution, equal to $\varphi_f - \varphi_e$;

φ — volume fraction of strong ("good") solvent in mobile phase; THF in this study;

φ_c — critical value of φ such that $k' = 1$; ref. 34, eqn. 8;

φ_e — value of φ at elution of band; band elutes in mobile phase of composition φ;

φ_f — final value of φ in gradient;

φ_0 — initial value of φ in gradient;

φ_s — value of φ that will just dissolve a sample of given size (weight) by a given volume of solvent;

χ_{1c} — critical solvent interaction parameter.

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