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CONVENTIONAL CHROMATOGRAPHIC THEORY *VERSUS* "CRITICAL" SOLUTION BEHAVIOR IN THE SEPARATION OF LARGE MOLECULES BY GRADIENT ELUTION

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SUMMARY

Two different theories of large-molecule gradient elution have been described in the literature: (a) a precipitation model and (b) conventional chromatographic retention involving sorption of solute molecules to the stationary phase. In the preceding paper it was shown that either model can apply under certain limiting conditions of sample size and polymer solubility in the mobile phase. An alternative description of these separations has been put forth by Armstrong and co-workers in terms of the "critical" solution behavior of large molecules; it is stated that conventional retention theory cannot explain these large-molecule separations. Evidence in support of "critical solution" theory is examined and compared with other data from the literature. It is concluded that previous arguments in support of unique effects due to "critical" solution behavior lack credibility, and that no evidence for such effects exist, at least for molecules smaller than 230 000 daltons.

INTRODUCTION

In the preceding paper[1] we presented a general model for the gradient elution separation of large molecules, one which recognizes the possibility of two different retention processes: (a) "normal" chromatographic retention (sorption of solute by the stationary phase) and (b) precipitation–redissolution. It was shown there that these two retention modes can be distinguished by (a) obtaining retention data as a function of sample size for a wide range of sample weights and (b) comparing these retention data with the solubility of the sample as a function of mobile phase composition (values of φ).

It was further shown for the reversed-phase gradient elution separation of a

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50 000 dalton polystyrene that "normal" chromatographic retention describes this system for sample weights less than 200 μg. It was also suggested that a sorption retention process may be the rule for other samples also, at least for the sample sizes (nanograms to micrograms) normally employed in high-performance liquid chromatography (HPLC). For samples that are relatively soluble in the mobile phase, such as proteins and other biomolecules, a precipitation process seems generally unlikely.

Armstrong and co-workers have published extensively[2–8] on the question of which retention process describes the gradient separation of a wide variety of synthetic polymer samples. For conditions where the sample size is small, and where "normal" retention by a sorption process would be expected, they propose that separation is dominated by what they describe as the "critical" composition of the mobile phase. It has also been claimed[2] that the gradient separation of large biomolecules requires "critical behavior" to be taken into account; however, they feel that the separation of proteins and other biomacromolecules may involve additional complexity beyond that of polymer separations.

We, on the other hand, have carried out detailed studies[9–15] which show that the separation of synthetic polymers, peptides and proteins by gradient elution (reversed-phase, ion-exchange or hydrophobic interaction chromatography) can be described quantitatively by a model based on small-molecule ("normal") chromatographic retention. There is no indication of any effects due to "critical behavior" as the sample molecular weight increases into the macromolecule range. The reasons for this differing interpretation of a broad set of experimental data seem to arise from certain misconceptions concerning conventional chromatographic theory, and from a lack of precision in defining various concepts. In this paper we attempt to clarify certain issues that have encouraged past controversy. Our hope is that future work will then be less likely to give rise to contrasting conclusions from the same data base.

THEORY*

The "critical behavior" model has been described in considerable detail[2–8], but (as we shall see) somewhat obscurely. It will therefore help first to review some essential features of conventional chromatographic theory and its practical assumptions. These aspects of conventional theory will then be compared with various claims on behalf of the Armstrong model.

Conventional model for large-molecule gradient elution

The main features of conventional theory have been reviewed[16–22] and will be taken as the starting point. It is assumed in the usual case that retention is governed by an equilibrium distribution of solute X between stationary (s) and mobile (m) phases:

$$X_m \rightleftharpoons X_s \tag{1}$$

* For a glossary of the symbols used, see ref. 1.

The rates of the forward and backward steps for the above process (interfacial mass transfer) are normally fast. Often a particular solute can exist in two (or more) forms or conformations (X and X*), leading to more complex retention equilibria:

$$X_m^* \rightleftharpoons X_m \rightleftharpoons X_s \rightleftharpoons X_s^* \tag{2}$$

As long as the rates of interconversion of X and X* in either the mobile phase or stationary phase are fast (and interfacial mass transfer is also fast), multiple solute forms have no detrimental effect on the use of conventional chromatographic theory. That is, this situation is equivalent to the case of a single (average) configuration of the solute molecule.

A further complication can arise if multiple retention processes are possible. For example, a given solute might be retained by a reversed-phase (hydrophobic) process on an alkyl bonded-phase, and by additional silanophilic or ion-exchange processes (involving silanol groups) on the same silica surface[23]. We can express these different states of the retained solute molecule as X and X#:

$$X_s \rightleftharpoons X_m \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_s^{\#} \tag{3}$$

Here rate constants k_1 and k_{-1} are indicated for the sorption–desorption of solute molecules X#. Again, the existence of multiple retention processes need not affect the application of conventional theory to the description of such separations. Thus, if i separate retention processes (#, eqn. 3) are involved, and the molecule X can exist in j different forms (*, eqn. 2), the overall value of k' for the sample X is

$$k' = \sum^{i,j} f_j \, k_{ij} \tag{3a}$$

where f_j is the fraction of molecules X in form j and k_{ij} is the k' value for retention of molecules in form j via process i. The potentially complex situation summarized by eqn. 3a is readily treated by conventional theory, using the average k' value given by this relationship.

However, a secondary retention process (eqn. 3) often involves small phase ratios, so that the column may become overloaded by even small solute concentrations. This then leads to tailing bands and k' values that change with solute concentration (see discussion in ref. 24). Also, the rate constants k_1 and k_{-1} may be small in secondary retention processes. This is equivalent to slow interfacial mass transfer, which can complicate (but not invalidate) the application of conventional theory with its usual assumption of fast mass transfer[16,25]. A related problem with similar consequences is the slow interconversion of separating species such as X and X* in eqn. 2 (see discussion in ref. 26).

A rigorous theory exists for the interrelationship of retention and band width for gradient and isocratic elution[21,22], starting with the basic equation

$$\int_0^{V_g} \mathrm{d}V/V_a = 1 \tag{4}$$

where V_g is the corrected retention volume of the band in a gradient run, V is the volume of mobile phase that has passed through the column at some time t and V_a is the instantaneous (corrected) retention volume for an isocratic run with the same mobile phase (as is in contact with the gradient band at time t). Eqn. 4 assumes that solute migration in gradient elution is equivalent to a series of infinitesimal migrations under isocratic conditions —the conditions that exist at the band center at any instant during gradient elution.

When gradient systems are of a special type, so-called linear solvent strenth (LSS) gradients, this theory reduces to fairly simple relationships that can predict every aspect of gradient separations (when data for corresponding isocratic separations are known). If linear gradients are used (the volume fraction φ of a strong solvent increases linearly with time during the gradient), then the LSS model for reversed-phase HPLC requires that isocratic retention be approximatable as

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

where k_w is the k' value for water as mobile phase and S is a constant for a given solute–mobile phase combination.

According to the theory of LSS gradients, the average or effective k' value of the solute during gradient elution is

$$\begin{aligned} \bar{k} &= (2/2.3)(t_G/\Delta\varphi\; S\; t_0) \\ &\approx t_G/\Delta\varphi\; S\; t_0 \end{aligned} \tag{6}$$

The value of S in eqn. 5 is of central importance to the present discussion. The assumption of a "normal" retention process that involves displacement of sorbed mobile phase molecules by a sorbing sample molecule leads[9,27,28] to

$$S \approx C\,x \tag{7}$$

for a polymer with x repeating units, or

$$S \approx C'\,M \tag{7a}$$

where M is the molecular weight of the polymer and C and C' are constants (only φ and M varying). If the sorption of all repeating units becomes more difficult as the size of the polymer increases (see discussion of Figs. 6–8 in ref. 29), or if a solute molecule retains some tertiary (three-dimensional) structure upon retention, then the dependence of S on M may be approximatable by some fractional power n:

$$S \approx C'\,M^n \tag{7b}$$

Experimentally, it is found that $n \approx 0.5$ for the reversed-phase HPLC separation of polystyrenes [tetrahydrofuran (THF)–water] or peptides and proteins (acetonitrile–water), as shown in Fig. 1. Note that there is no discontinuity in the plots of Fig. 1 over the molecular weight range $1 \leqslant M \leqslant 233$ kdaltons. This fails to confirm any major change in the retention process (*e.g.*, the transitional behavior referred to in ref. 2) within this range of polymer molecular weights.

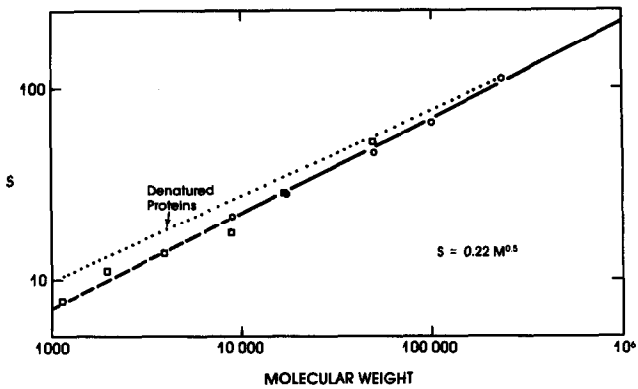

Fig. 1. Dependence of solute S values $[-\mathrm{d}(\log k')/\mathrm{d}\varphi]$ on solute molecular weight (reversed-phase). Solid line, data for polystyrenes with THF–water as mobile phase; ○, data from ref. 45; □, data from ref. 29. Broken line, data for peptides and proteins with acetonitrile–water mobile phase[10].

Armstrong model for large-molecule gradient elution

A good theoretical basis exists for calculating polymer conformation as a function of mobile phase composition plus the molecular weight and concentration of the sample. This in turn allows development of a theory of polymer sample gradient elution based on "critical" solution behavior[7,8]. According to this theory,

$$k' = \exp\left[Ax(\varphi - \varphi_c)\right] \tag{8}$$

where A is a negative constant for polymers with molecular weights greater than 10 000 daltons, x is the degree of polymerization (proportional to molecular weight) and φ_c is the "critical" value of φ such that $k' = 1$ (ref. 30). Because polymers involve large values of x, the factor Ax is large for high-molecular-weight samples, and plots of log k' *vs.* φ become fairly steep —effectively infinite in practical terms[2]. The alleged consequences for chromatographic separation then include the following:

(1) k' ranges from zero to a large value for $\varphi = \varphi_c \pm \delta\varphi$, where $\delta\varphi$ is small in practice; precipitation of the polymer often occurs within this compositional range $(\varphi_c \pm \delta\varphi)$;

(2) in gradient elution, the solute (polymer) remains at the column inlet until mobile phase of "critical" composition (φ_c) overtakes it, then the polymer "pops off" the column packing and moves through the column with $k' = 0$ during elution;

(3) under isocratic conditions, the attainment of finite, non-zero values of k' is effectively impossible; the polymer will either be strongly retained or elute with $k' = 0$.

There are a number of other consequences of "critical" solution behavior to which we shall return.

Is there really a difference between "critical behavior" and the logical extrapolation of small-molecule gradient elution?

Eqn. 8 can be restated in the same terms that we have used for reversed-phase separation, if we replace Ax with $-S$, and if S is roughly proportional to x (eqn. 7):

$$k' = 10^{(1/2.3)Ax(\varphi - \varphi_c)} \tag{9}$$

or

$$\log k' = -(1/2.3)(AM\varphi_c) - S\varphi$$
$$= \text{constant} - S\varphi \quad (10)$$

which is of the same form as eqn. 5. We have noted previously[29] that S can be large for polymers with molecular weights above 10 000, so that most of the consequences of "critical behavior" are predictable by extrapolating small-molecule theory for application to large solute molecules. The main difference relates to whether these effects are continuous as in normal chromatography, or discontinuous as implied in ref. 2, and whether values of S are just large, or effectively infinite. We shall see that precise retention data are required to make these distinctions.

The Armstrong model (which we shall elaborate on shortly) has been claimed to differ substantially from the model assumed in "normal" chromatographic separation. It should therefore be easy to compare these two models with relevant experimental data, and to conclude which one is more likely. For us, however, this comparison has been complicated by ambiguity in the terminology and scope of the Armstrong model (*e.g.*, ref. 2). The original "critical behavior" model[7,8] describes the conformation of an isolated flexible polymer chain on a plane surface, *i.e.*, sorption as opposed to precipitation*. We therefore assume that Armstrong and co-workers are discussing sorption when they talk of "critical behavior". This assumption appears to be contradicted, however, by the redifinition in ref. 2 of "critical behavior": "roughly ... the composition of a binary solvent system that will just dissolve an immobilized polymer or just precipitate a dissolved polymer in the presence of the stationary phase". The impression that Armstrong and co-workers now consider precipitation as part of "critical behavior" is further strengthened by their inclusion of (a) the Glöckner precipitation model (see ref. 1 for a review) plus (b) other polymer fractionations based on solubility in their most recent review[2]. We therefore are not sure of Armstrong and co-workers' current, exact meaning of "critical behavior".

The data reported by Armstrong and co-workers in support of "critical behavior" actually appear to be consistent with classical precipitation (see refs. 31 and 32 and references cited therein) and the model presented in our first paper[1]. Thus many of the "unique" observations attributed to "critical behavior" were described many years ago for precipitation separations. For example, column fractionation of polymers by fractional precipitation–dissolution is insensitive to column length[33]. Likewise, separations based on precipitation should not be influenced by the type of column packing or the time of sample injection (relative to initiation of the gradient), and isocratic elution should not yield normal elution bands[1]. These conclusions for precipitation processes, however, are not necessarily true for separations under small-sample (sorption) conditions, as we shall attempt to point out.

RESULTS AND DISCUSSION

We wish to assess the arguments of Armstrong and co-workers with regard to

* In subsequent discussions with Boehm and Martire, these workers made clear that their work refers only to the small-sample case, where polymer precipitation as in ref. 1 is not involved. There is also nothing in the "critical behavior" treatment in refs. 7 and 8 to suggest discontinuous behavior at molecular weights > 10 000 daltons, as suggested in ref. 2.

"critical behavior" *versus* "small-molecule" theory plus the use of the LSS model. In the following discussion, we treat the case of small-sample separations (sorption as opposed to precipitation). In view of the ambiguity that exists over whether "critical behavior" may mean precipitation rather than sorption, however, see the further discussion in Appendix I.

It is useful to organize the following discussion according to the separate parts of an overall model of these separations:

(1) What is the relationship beween k', φ and M under equilibrium (isocratic) conditions, *e.g.*, eqn. 5, eqn. 8 or some other equation?

TABLE I

SUMMARY OF THE VARIOUS FEATURES AND SUPPORTING CLAIMS FOR THE "CRITICAL BEHAVIOR" MODEL OF LARGE MOLECULE SEPARATION USING GRADIENT ELUTION

No.	*Feature*
1	When narrow-pore packings are used, solute molecules are excluded from the pores, and move through the column more rapidly than solvent molecules.
2	High polymers show no significant difference in retention time or band width when different stationary phases (C_2, C_3, C_8, C_{18}) are used. This presumably proves that there is no significant interaction between the solute and the stationary phase[5,46].
3	"... A polymer tends to change its size and shape in response to ... mobile phase composition"; a concept "... which is foreign to traditional chromatography"[2].
4	While eqns. 4 and 5 may be appropriate to describe the retention of small molecules, the correct expression for homopolymers in gradient HPLC is eqn. 8. "An examination of isocratic and gradient retention data for various polymers allows testing and comparison of (these two equations)"[2,5].
5	"Traditional chromatographic theory is ... thermodynamically incorrect for macromolecules", "... it does not consider ... segment–segment interactions and local entropic contributions to the free energy of the system..."[2].
6	"If in fact traditional ... theory plays a role in the separation of ... proteins, it would be one of several factors that are operative. In fact, a dual retention mechanism was postulated by Horváth..."[2].
7	Isocratic elution of macromolecules gives either strong retention ($k' \approx \infty$) or no retention ($k' \approx 0$), depending on whether $\varphi < \varphi_c$ or $\varphi > \varphi_c$, respectively. Elution bands with good peak shape are not observed, except for $k' = 0$[2,46].
8	"... (Polymer) peaks will tend to be compressed in the later eluting high MW region"; a concept "... which is foreign to traditional chromatography"[2].
9	"... A greater change would be observed in the k' of a polymer with changing temperature than in that of a small solute"[2].
10	"One can obtain ... better resolution for these solutes (polymers) using shorter columns", a "surprising result"[6].
11	"The slope of these lines (S) is nearly infinite for practical purposes. This tends to make the application and use of eqns. 5 and 6 untenable"[2].
12	The "normal" *vs.* "critical behavior" models of polymer retention can be clearly distinguished by changing the injection time for some fixed gradient interval (so that injection does not coincide with the start of the gradient). The "critical behavior" model predicts that injection time will have no effect on gradient retention time of the polymer sample. Similar arguments for the TLC separation of polymers can be developed, where the initial spot position on the plate replaces the injection time[4].
13	"... One of the fundamental considerations of (the small-molecule approach) is the linear relationship between k' and φ (*sic*)". This is generally not observed, and this obviates the applicability of the LSS approach[2].
14	"Mass transfer ... is negligible for high MW polymers in this particular form of chromatography"[6].

(2) What is the relationship between gradient and isocratic retention? Can we assume that eqn. 4 applies?

(3) If the LSS model is used for relating gradient and isocratic retention, what errors will result from the use of eqn. 5 as an approximation to the exact dependence of k' on φ? How do these errors affect the prediction of separation as a function of gradient conditions?

(4) What other observations can provide additional checks to confirm the "best" model?

Table I summarizes several claims in support of the "critical behavior" model, organized according to the preceding four areas plus "sorption *vs.* precipitation" (Appendix I). The following sub-sections and Appendix I are based on Table I, with numbering within each sub-section corresponding to that in Table I.

Relationship of k' with φ and M

Polymer molecules can change shape as the mobile phase composition is varied (No. 3). Changes in molecular shape as the mobile phase composition varies probably occur for a variety of both large and small molecules. With small molecules, these changes in shape generally have little direct effect on the separation, or their effects may be difficult to recognize. However, such effects are not unknown, as seen in the classic study of Melander *et al.*[34]. They showed striking effects due to changes in the molecular conformation of oligo(ethylene glycol) derivatives of molecular weight 200–600 daltons, with change in molecular shape being induced by changes in mobile phase composition or temperature. However, the importance of these effects was seen mainly in peculiar changes of retention *vs.* solute structure (irregular change in retention as temperature or φ was varied). Apart from these effects, normal chromatographic theory was adequate to describe retention, and these specific effects were also adequately described in terms of conventional theory.

Provided that the interconversions between different conformations of the solute molecule (eqn. 2) are fast, classical theory has no difficulty in treating this situation. The main problem occurs when the interconversions occur on a time scale that is similar to that of chromatographic retention[26]. In this instance a real problem can arise in applying simplified conventional theory, especially for large molecules. For example, proteins and other biological macromolecules commonly exist in various discrete conformations (native *vs.* denatured), and the rates of interconversion (denaturation or refolding) are often similar to those for chromatographic separation[35]. This has led to reports of a number of "strange" separations of these compounds by reversed-phase gradient elution[36–39]. For example, bands are anomalously broad or misshapen, and two or more peaks in the chromatogram may represent a single compound. In other instances, initial separation of a protein by gradient elution may result in elution of the sample as a distinct, well behaved band, but with incomplete recovery. Subsequent gradients (without injecting sample) then show elution of the compound in decreasing amounts, but with a normal band eluted at the same retention time[40]. In each of these instances, slow interconversion of different conformations of the protein probably contribute to the observed anomalies.

These slow conformational interconversions do not represent a "failure" of traditional chromatographic theory, but they do complicate its application to systems which exhibit these effects. Further, the resulting separations are often poor. We

believe that the solution to this problem is to begin with conventional chromatographic theory (and the quantitative LSS model that is derived from this theory). When such effects are suspected, experimental band widths can be compared with band widths predicted by the model[11,12,14,15]. If slow interconversion is confirmed, than attempts can be made to speed up the kinetics of interconversion so as to improve the separation and bring the experimental results into agreement with data calculated from the LSS model.

In any case, the "critical behavior" model[7,8] does not even consider such kinetic effects during separation. Therefore, such effects cannot be cited as evidence for "critical behavior" *vs.* conventional retention.

Retention eqns. 5 and 6 are applicable only for small molecules; eqn. 8 applies for macromolecules (No. 4). Much has been made by Armstrong and co-workers (*e.g.* ref. 2) of the difference between eqns. 5 and 8. There are two issues here: first, which equation is experimentally more reliable and second, which equation allows a more detailed prediction of chromatographic behavior from the physical properties of the chromatographic system? Eqn. 5 is an empirical relationship that is in reasonable agreement with a large body of data for large and small solute molecules. Therefore, its use in a particular situation should lead to reliable predictions of retention, provided that S is known and there are "no anomalous" effects of the type discussed in the preceding section (No. 3). Eqn. 8 is derived from a detailed statistical thermodynamic analysis of polymer retention in simplified chromatographic systems. In principle, it should allow accurate predictions of polymer retention as a function of φ and M without the need for empirical parameters such as S and k_w. However, this treatment is limited by the fact that it assumes an equilibrium distribution of chain segments at a plane surface, rather than in a pore space.

Although the model in refs. 7 and 8 is an encouraging first attempt, we feel that (a) this model needs further development, similar to that presented for adsorption in parallel plates[41] and cylindrical pores[42], and (b) the issue of non-equilibrium separation must be addressed before any precise predictions of retention from the physical properties of the system can be realized.

As eqn. 5 represents an empirical summary of actual experimental data, and as eqn. 8 is theoretically based, it is not surprising that they are found to be equivalent in form (eqns. 9 and 10). The major difference between eqns. 5 and 8 is the value of n in eqn. 7 b, which is experimentally equal to 0.5, while the implicit value of n in eqn. 8 is 1.0. That is, $d(\log k')/d\varphi$ is predicted to be proportional to M by eqn. 8, and to $M^{1/2}$ by eqn. 5. The reason for this discrepancy may lie in the assumption of adsorption onto a plane surface (see above), but we do not know at this point. Experimental data verifying the direct proportionality between $d(\log k')/d\varphi$ and M have not been reported. In any case, the assumption that eqn. 8 is correct does not invalidate eqn. 4 as has been claimed[2,5].

Traditional chromatographic theory is thermodynamically incorrect for macromolecules (No. 5). Most of traditional chromatographic theory treats the consequences of segment–segment interactions, local entropic contributions, etc. That is, the thermodynamic description of large-molecule systems is different in important respects to that of small-molecule systems[43], but the final dependence of k' on mobile phase composition is what chromatographers and chromatographic theory often begin with. This is the case for previous applications of the LSS model, which simply

assumes that k' is some function of experimental conditions and is experimentally determinable. Treatments such as those in refs. 7 and 8 which are intended to clarify the dependence of large-molecule retention on experimental conditions should prove useful in better understanding and controlling the separation of macromolecules. However, these treatments have so far not called into question the applicability of conventional theory once a relationship between k' and φ has been established.

There are further consequences of large-molecule thermodynamics, however. Thus, the equilibrium constant can vary with flow-rate, a result not anticipated in small-molecule theory. However, this is generally a second-order effect that is much less important than the primary separation variables treated by classical theory (*e.g.*, see discussion of k' changes with flow-rate for insulin[10]). Until data are presented showing the practical importance of such flow-rate effects, we should continue to emphasize other ("normal chromatographic") aspects of these large-molecule separations.

Dual retention mechanisms complicate the application of small-molecule theory to many systems (No. 6). This argument was considered earlier in the Theory section. There is no inherent problem in a dual-retention process for a given separation system. However, phenomena of this type can result in poor chromatographic separations and make the prediction and control of chromatographic separation difficult. Practical chromatographers generally try to suppress secondary retention processes, such as silanol interactions in reversed-phase separations. An example is the use of triethylamine as a mobile phase additive to reduce band broadening and tailing in the reversed-phase separation of peptides[44].

Present small-molecule theory can still be useful in the case of poor chromatography due to secondary retention, just as for the problem of slowly interconvertering conformations of large molecules (No. 3 above). Thus, the LSS model allows the prediction of band widths in the absence of secondary retention effects that lead to poor chromatography[12]; comparison of experimental values with calculated band widths can then confirm the presence of anomalous band broadening, leading to its correction by well known means.

Relationship between gradient and isocratic retention

This area seems to involve the most confusion in comparisons of "normal" *vs.* "critical behavior" chromatography. Eqn. 4 is the basis for all calculations of gra-

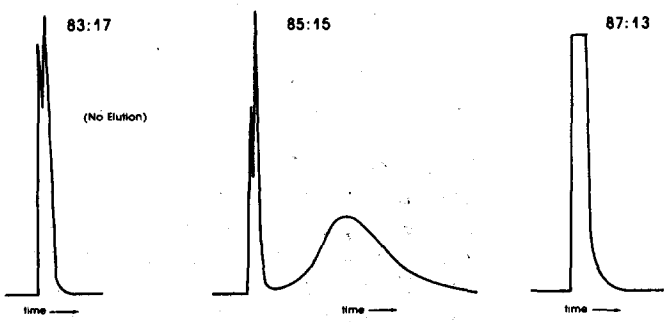

Fig. 2. Isocratic elution of 50 000-dalton polystyrene sample from a C_{18} column by different concentrations of tetrahydrofuran–water[29].

dient retention based on small-molecule theory. It has been verified on numerous occasions for small molecules[21,22] and for large molecules[10,15,45]. Discussions of "critical behavior" have ignored this relationship, presumably because eqn. 4 simplifies to elution of the solute at a mobile phase composition φ_c, when S is effectively infinite. What this means is that both conventional theory and "critical behavior" predict the same dependence of large-molecule retention on gradient conditions, as a first approximation. In order to distinguish between these two models, we shall show that it is necessary to refine the precision of retention measurements by one or two orders of magnitude, compared with the usual correlations of ±1% (*cf.*, Fig. 4 in ref. 1).

Isocratic elution of polymer solutes generally gives anomalous results (No. 7). If the "simple" Armstrong model applies strictly, it would predict that isocratic bands with $k' > 0$ should not be observed; "... isocratic elution tends to either elute all of the macromolecules at once or not elute any of them"[2]. It appears that they are inclined to explain isocratic elution bands for polymers (when they are observed) in terms of a "transition region" between small and large-molecule chromatography, with the transition occurring for polymers of molecular weight greater than 10 000 daltons[5,46].

We have reported "regular" chromatographic bands for the isocratic elution of a 50 000-dalton polystyrene[29,45], as illustrated by the example in Fig. 2b. There are two reasons why others may have had difficulty in similarly showing "regular" elution behavior for high-molecular-weight polymers under isocratic conditions. First, the large values of S for these samples (eqn. 4) lead to rapid changes in k' for small changes in φ. This is illustrated in Fig. 2, where a 2% (v/v) change in THF concentration (0.02 in φ) leads either to no apparent elution [83% (v/v) THF, Fig. 2a] or immediate elution [87% (v/v) THF, Fig. 2c]. However, when smaller changes in φ are made, it is possible to observe k' values that fall in the range 1–10 (refs. 29 and 45).

A second reason for failing to observe "regular" elution bands in the isocratic separation of large polymer molecules (especially for molecular weights over 50 000 daltons) is that these samples are not homogeneous. Even very narrow polymer standards will usually have a distribution of polymer molecular weights of the order of ±10%. This in turn means that the retention of larger polymer molecules can be much greater than that of smaller molecules in the same sample. The polymer sample band width is then determined not by the width of individual compound bands (as in chromatographic separations), but by the envelope of retention times for individual components of the sample, as illustrated schematically in Fig. 3. This (potentially) extreme widening of the polymer band under isocratic conditions (with $k' > 0$) then makes the band indistinguishable from the baseline. We have observed this on repeated occasions in the attempted isocratic elution of polystyrenes with molecular weights of 100 000 and 233 000 daltons. In no instance have we observed elution bands except with $k' \approx 0$ (however, at these molecular weights and concentrations we could also be in the precipitation regime).

Polymer peaks tend to be narrower in the later part of the chromatogram (No. 8). If a series of polymer fractions of varying molecular weight are separated by gradient elution, it is commonly observed that later bands (of higher molecular weight) tend to be narrower, relative to earlier bands. This is seen by Armstrong and

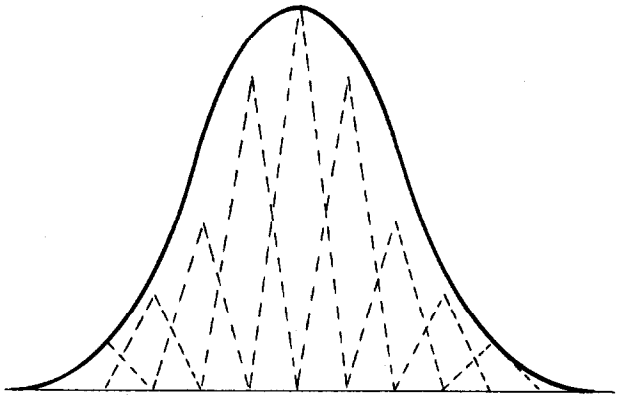

Fig. 3. Polymer elution band as the envelope of bands for individual oligomers.

Boehm[2] as "foreign to traditional chromatography". Two points need to be made here. First, in isocratic elution the normal pattern is usually an increase in band width with increasing retention, due to the consequences of conventional theory. Application of the same (conventional) logic to gradient separations[21] predicts that band widths will normally be roughly constant from one end of the chromatogram to the other, but it is possible to see regular (and predictable) increases or decreases in band width with increasing retention (*e.g.*, Fig. 22 in ref. 21 and related discussion).

Second, band width in these gradient polymer separations is controlled more by retention range, rather than by the width of individual oligomer bands (Fig. 3 and No. 7 above). The retention-time difference between adjacent oligomers decreases with increasing φ[29], which in turn correlates with later elution in the gradient. This compression of the retention range is probably the main reason for narrower bands at the end of the chromatogram in gradient separations of polymer samples. Certainly there is no reason to require any "unusual" explanation, or to invoke some new retention process ("critical behavior"). It should be mentioned, however, that precipitation separations may show further differences in band width *vs.* band position in the chromatogram.

Polymer retention changes more with temperature than for the case of small molecules (No. 9). This is hardly unexpected, as discussed in ref. 29. Retention is the result of a certain standard-state free energy of retention, which in turn is the sum of retention enthalpy and entropy terms. A polymer molecule on retention will generally pass from a random coil to a more ordered state, meaning that retention entropy will increase relative to the retention of a small molecule. As the free energy must remain similar for large and small molecules if k' values are to be similar, this means that the retention enthalpy for the polymer molecule must be relatively greater. This in turn means a greater temperature coefficient of retention.

Shorter columns give better separation in polymer gradient elution (No. 10). A number of workers have observed for gradient separations of proteins that the length of the column seems to make little difference to sample resolution[47–50]. The reason for this is that in gradient elution, a change in column length (which increases reso-

TABLE II

CALCULATED RESOLUTION AS A FUNCTION OF COLUMN LENGTH

From data in ref. 5.

Column length (cm)	*Relative resolution for different polymers**				
	9/35	*35/100*	*100/390*	*390/900*	*Average*
25	1.5	3.5	2.1	1.2	2.1
10	2.2	1.5	3.7	0.6	2.0
5	2.3	2.6	2.5	1.0	2.1
4	2.3	2.8	2.5	1.1	2.2

* 9/35 refers to resolution for 9000- and 35 000-dalton samples, respectively; 35/100, etc., have similar meanings.

lution) is offset by a corresponding decrease in the average k' value (as a result of changes in column length; eqn. 6). This can result in a decreased dependence of separation on column length in large-molecule gradient elution[9]. Other studies[12] have shown that column length can be important in affecting such separations, and in any case the column appears to play a similar role in these separations to that in isocratic separations of small molecules.

For polymer separations, where the band width is controlled by the retention range (No. 7 above), a different situation prevails. An increase in column length will affect the band widths of individual oligomers, but this will not in turn have much effect on polymer-fraction band width. A similar situation has long been known for the separation of compound classes by adsorption chromatography[51]. Here each compound class is composed of many individual compounds of varying retention, and an increase in column plate number has little effect on the relative separation of adjacent compound classes. In any case, the observation (for polymer separations by gradient elution) that column length has little effect on separation is not surprising.

The specific claim[5] that separation is actually better on short columns is not borne out by the data reported there. Relative values of resolution can be calculated from these data by dividing the difference in retention volumes for two adjacent polymer bands by the average band width. The results in Table II are obtained. Within experimental error, the average resolution values appear identical for each column.

The slopes S of plots of log k' vs. φ are effectively infinite for large polymers (No. 11). There is little disagreement over the fact that plots of log k' *vs.* φ become steeper for larger polymer molecules. The value of S from eqn. 4 is given for polystyrenes separated by reversed-phase systems and THF–water mobile phases (Fig. 1) as

$$S = 0.22\, M^{1/2} \tag{10}$$

From this we can calculate that S will equal 70 for a 10^5-dalton polystyrene and 220 for a 10^6-dalton sample. For a 10^6-dalton sample, this means that a 1% (v/v) change in mobile phase composition will cause k' to change by a factor of about 200. This

can be restated as saying that the slopes of plots of log k' *vs.* φ are steep. It does not follow, however, that the slope is infinite, or that any special effects result from slope steepness. The theory of gradient elution as expressed in the LSS model defines the role of gradient steepness in terms of eqn. 6. A large value of S therefore means a small value of $\bar{k}$, which can adversely affect separations. However, good chromatographic practice for this situation suggests simply increasing the gradient time, t_G, or decreasing the gradient range, $\Delta\varphi$, in order to compensate for large S values and maintain values of $\bar{k}$ within a practical range (usually $1 \leqslant \bar{k} \leqslant 20$). Under these conditions, theory predicts no ill effects from large S values, and certainly there is no basis to anticipate that the theory of gradient elution will break down simply because of large S values.

Direct experimental confirmation of the elution of large molecules (proteins) in gradient elution with $\bar{k} > 0$ has recently been reported[52]. DiBussolo and Gant[52] used glass columns and colored compounds to observe protein migration during reversed-phase gradient elution. They found that bands moved through the column at a slower rate than did the mobile phase, and the shape of these migration *vs.* time curves was similar to that predicted by the theory for "normal" chromatography (*cf.*, Fig. 7 in ref. 52 *vs.* Fig. 8 in ref. 9).

Sample injection time in polymer gradient elution does not affect retention time (No. 12). It is observed that changing the time of sample injection (after the start of the gradient) does not affect the final retention time of a large molecule, *i.e.*, the band leaves the column in mobile phase of composition φ, where $\varphi \approx \varphi_c$ (independent of injection time). A similar result is observed for separations by gradient thin-layer chromatography (TLC); this behavior has been claimed to show that "critical behavior" is applicable in these separations[4]. The same result is predicted by classical theory, however, as we shall show next for gradient elution in columns.

With large-molecule solutes, the value of S will be fairly large, as is the value of k' during the early stages of the gradient. Under these conditions the sample band remains at the column inlet until its k' value drops below a value of about 100. It then begins to migrate through the column, leaving the column generally with a k' value of 0.5–10 (depending on gradient steepness and the value of S; see eqn. 6). This means that the change in k' from the beginning of band migration until it leaves the column will be about 100-fold at most, corresponding to a change in φ of $\log(100)/S$ (see eqn. 5). Now, values of S for large molecules are typically of the order of 100, so that the change in φ (at the band center) during migration of the band through the column is about $\log(100)/100 \approx 0.02$. For a 0–100% gradient, this corresponds to a time of $0.02t_G$. This means that injection of the sample at any time during the gradient, but at least $0.02t_G + t_0$ before elution of the band, will not affect its retention time. For a further discussion of this point, see pp. 5–6 in ref. 53.

A similar situation prevails for gradient TLC. Again, the band will migrate along the plate with a k' value in the range 1–100, corresponding to a range of φ values of about ± 0.01. When the sample is spotted higher on the plate, instead of at the usual position at the bottom of the plate, the sample simply waits until mobile phase of the right composition (for k' less than 100) reaches the sample. The sample band then migrates along the plate in the usual manner. An exact treatment of this behavior is possible but unprofitable, because of imprecision in our ability to define the gradient for these TLC separations.

Effect of deviations from eqn. 5 on LSS theory

The accuracy of the LSS model as an approximation to eqn. 4 is really not related to the issue of the "normal" *vs.* "critical behavior" models of macromolecule gradient elution. However, predictions based on the LSS approximation have been used to discuss the "critical behavior" model. For this reason it is important to address this issue.

Linear plots of log k' vs. φ are generally not observed, making LSS theory inapplicable (No. 13). This issue has been treated at length[4,5]. It has been shown that LSS theory can readily be extended to treat separations where log k' does not change linearly with time during the gradient, as required originally for LSS systems. Therefore, for linear gradients and non-linear plots of log k' *vs.* φ, the original premise of LSS gradient elution need not be strictly observed, and the practical consequences are negligible. Small errors in prediction of separation can result in such cases, but the magnitude of these errors can be calculated and corrections applied for exact agreement with experiment*. As an example, LSS theory was applied[4,5] to the isocratic and gradient separation by reversed-phase HPLC of polystyrenes ($9000 \leqslant M \leqslant 233\,000$), with excellent agreement between experimental and gradient-derived values of k' for isocratic separation.

Other tests of "normal" vs. "critical behavior" models

We have noted that predictions of retention by these two models are in general similar. Experimental data can be used to differentiate which model applies in a given system, but usually precise values of φ_c are required. A more comprehensive test of how well each model predicts experimental results can be found in studies of mass transfer or band broadening. Such studies have been ignored by Armstrong and co-workers, but have been the primary focus of our group over the past 3 years.

Mass transfer of solute molecules does not contribute to separation of polymers by gradient elution (No. 14). The assumption that mass transfer is not involved in the Armstrong model of polymer separation is at first difficult to understand, particularly as no alternative description of the kinetics of the "critical behavior" process has been offered in its stead. Some insight can be obtained from the discussion of the preceding section, where we pointed out that band width in these polymer separations is a result largely of the retention range of the sample, rather than the band width of individual compounds. That is, partial separation of the components of the polymer occurs during elution of the sample. Bui *et al.*[5] recognize this effect for lower molecular weight polymers, but reject its applicability with larger polymer molecules. This then leaves the question of what does cause band broadening with these samples.

There is little doubt that some kind of mass transfer effects must be present. The kinetics of the retention process (whether normal chromatography, precipitation or "critical behavior") must in some way affect the band widths of individual compounds in the sample. Because the retention range of a polydisperse sample normally

* This is true even for biphasic plots of log k' *vs.* φ (*e.g.*, ref. 54), where k' first decreases with increasing φ then increases for even larger values of φ. In all such situations documented to date, minimum values of k' are usually much less than one. In this instance, and for reasonable gradient conditions, the compound is then eluted before values of k' have any change to reverse. During migration of the solute band through the column, plots of log k' *vs.* φ will therefore be nearly linear for reversed-phase systems.

determines the band width (Fig. 3), the separation of polymer samples tells us little about the nature and magnitude of mass transfer effects involved. On the other hand, a study of mass transfer or band broadening for individual macromolecular species (*i.e.*, compounds) can shed a good deal of light on the nature of retention in these separations. Naturally occurring monodisperse biopolymers provide us with this opportunity, and elsewhere[11,14,15] we have examined band broadening for the gradient separation of peptides and proteins with molecular weights as high as 160 000 daltons. These results appear to be in complete agreement with conventional chromatographic theory and the LSS model that is based on this theory. Specifically, it is possible to predict how the band width will change as the separation conditions are varied, with an accuracy of about ± 10–20% for changes in band width by a factor of over 10. These findings represent an important further argument for "normal" chromatography as opposed to "critical behavior" in the gradient separation of large molecules.

Various other claims of "chromatographic uniqueness' are implied in the work of Armstrong and co-workers[2–8] for the gradient separation of polymers. We find these equally unconvincing as regards a need to postulate "critical behavior" for these separations.

CONCLUSIONS

Practical separations of macromolecules using gradient elution are of increasing interest, and detailed quantitative theories are required to better understand and use these procedures. Recently two different views of these gradient separations (for small samples) have been advanced: (a) "critical solution behavior" explains completely the retention of polymer samples, and significantly affects retention for biological macromolecules as well, or (b) retention is governed by the same processes that hold for small molecule separations. The question of which model is closer to experimental reality is an important one, in order that we can more reliably vary the separation conditions for an optimum result. We have reviewed the various arguments on behalf of "critical solution behavior" in detail, and have compared these with corresponding predictions of the "small-molecule" or "normal chromatography" model. Our conclusions are as follows:

(1) It is not clear from the "critical behavior" model whether sample retention occurs by a sorption or precipitation–redissolution process. We assume that sorption is intended, but the alternative possibility is treated in the preceding paper[1].

(2) Further development of the "small-molecule" model leads to an expanded treatment that includes macromolecules and that permits quantitative predictions of retention and band width as a function of solute molecular weight and separation conditions. Comparison of this expanded model with the "critical behavior" model shows similar predictions by each model for solute retention in macromolecule gradient elution.

(3) The essential difference between the two models involves the rate of change of sample retention with change in mobile phase composition φ: $d(\log k')/d\varphi = S$ in the present nomenclature. Both models predict large values of S for macromolecular solutes (both polymers and proteins) separated by reversed- or normal-phase HPLC; however, the "critical behavior" model assumes that above some minimum

solute molecular weight (> 10 000 daltons) the values of S are so large as to be effectively infinite. This means that elution of the solute occurs in a "critical" mobile-phase composition φ_c that is independent of conditions. The "normal chromatography" model predicts that this is only a first approximation, and that careful measurements of retention time will show that the solute band elutes at values of φ that vary (slightly) with experimental conditions.

(4) The main distinction between the two models is that the "critical behavior" model cannot predict band width, a very important parameter in optimizing separations of the present kind. The "normal chromatography" model with the use of the linear solvent strength (LSS) theory has so far been reasonably successful in this regard.

Overall we can say that the "critical behavior" model represents a first approximation for understanding solute retention in the separation of macromolecules by gradient elution, particularly for the case of polymers *vs.* that of individual macromolecular compounds (*e.g.*, proteins). However, it does not contradict (or add to) the theory previously developed for the chromatographic separation of both large and small molecules, and it is less precise and general than that theory. The concept of "critical behavior" as a phenomenon that replaces normal chromatography when solute molecules exceed some minimum size is not supported by available evidence. The same basic rules appear to apply to the separation of both large and small molecules.

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APPENDIX I

Sorption versus precipitation?

Solute molecules are excluded from small pores (No. 1). The degree to which solute molecules penetrate small pores varies with the mode of separation (precipitation or sorption) and the enthalpic attraction between polymer molecules and the stationary phase. It is proposed for reversed- or normal-phase separation (as distinct from size-exclusion chromatography) that large polymer molecules cannot penetrate into small pores, but are excluded by a size-exclusion process. At first this seems reasonable, as it is just what occurs if a strong solvent is used so that active retention on to the surface of the stationary phase is eliminated ($k' = 0$). However, when dealing with random-coil polymeric solutes, it should be appreciated that the "size" of the molecule for size-exclusion separation is determined by the hydrodynamic diameter of the coil. Entry of the molecule into a small pore can still occur if the coil unravels or distorts.

In size-exclusion chromatography there is little incentive for the molecule to do this, but in other (retentive) processes where k' can be large for a polymer solute, this strong (enthalpic) retention can overcome the entropic constraint of a large coiled polymer within a pore of small diameter[5,6]. It can in fact be shown that under ad-

sorptive conditions the probability of the molecule being found inside the pore is actually unity[41,42,56,57]. Additional examples have been given for the passage of very large macromolecules through membranes whose pore diameters are considerably smaller than the polymer-coil radius of gyration. Concentration difference and/or flow (rather than adsorption) provides the free-energy incentive to overcome entropic constraint of the polymer molecule from the pore[58,59].

In the light of the large body of experimental evidence and established theory from several related areas in polymer physical chemistry, the question in polymer chromatography (with strong adsorptive attraction) is not whether polymer chains penetrate small pores, but whether they do so completely in the time frame of the chromatographic experiment. This question has yet to be resolved for specific polymer chromatography systems. At least four cases exist, however, in addition to polymer sorbed from solution: (1) weak attraction of solubilized polymer to the surface of porous media and (2) strong, (3) weak or (4) no attraction of precipitated polymer to the stationary phase surface. Numerous examples of case 1 have been given, *e.g.*, "adsorptive gel permeation chromatography", where steric exclusion reduces the effect of adsorption[60,61]. In this case, enthalpic attraction is generally weak and pore penetration is incomplete. At the other extreme, the pore volume at the column head may not be penetrated significantly (or at all) if the polymer precipitates under the initial gradient conditions, as in cases 2, 3 and 4. Once resolubilized, the polymer will penetrate the pores completely if k' is large, partially if k' is small and to the extent predicted by normal size-exclusion chromatography if $k' = 0$.

There is a simple way of determining whether large polymer molecules are excluded or not excluded from small pores in a separation process such as reversed-phase HPLC. One can compare the relative retentions (k' values) of a series of polymers of different molecular size on packings of different pore size. If it is assumed that exclusion occurs (as measured by retention measurements under size-exclusion conditions, $k' = 0$), then the relative retention on different packings should vary predictably with solute molecular size. If it is assumed that exclusion does not occur, a different dependence of retention on sample molecular weight and packing pore size results. These comparisons have been made[29] for polystyrenes of molecular weight 208–50 000 daltons separated by reversed-phase HPLC with tetrahydrofuran–water as mobile phase. The clear conclusion from these studies is that exclusion under these conditions was generally unimportant.

This conclusion is strengthened by subsequently obtained data that allow a better estimate of the surface areas of the column-packings of ref. 29 (see Appendix II). If exclusion of the 17 500-dalton polystyrene does not occur for the 6–100-nm pore packings described in ref. 29, then the calculated stationary phase volume, V_s, (*i.e.*, the phase ratio from k' measurements) should correlate with the corrected surface area of these packings. This relationship is tested in Fig. 4 and seen to describe the actual experimental data.

Chemical nature of the column packing does not affect polymer retention times (No. 2). In the "critical behavior" model it is assumed that the stationary phase is actually an adsorbed layer of the least polar solvent on the reversed-phase particle[8,9]. This simplifies considerably the derivation of the capacity factor. In the light of the above assumption, it is not entirely clear that the "critical behavior" model can predict the effect of column type on retention. This was tested[6], however, for poly-

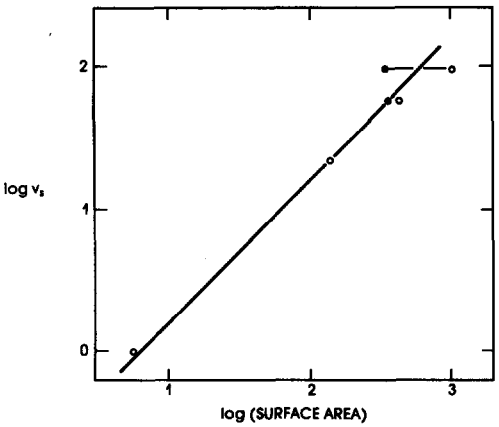

Fig. 4. Relative values of stationary phase volume, V_s (ratio to value for 100-nm pore packing), correlated with column surface area. Values of V_s derived from relative retention of polystyrenes (200–17 500 daltons) on these columns[29]. ○, Based on silica surface area; ●, based on area available for adsorption of acetonitrile[55].

styrene samples and columns with cyano, C_2, C_3, C_8 and C_{18} packings. It was claimed that, "As predicted, the high polymers showed no significant difference in retention volume ...".

We suspect that the data in ref. 6 were actually obtained in a precipitation mode, in which case there should not be any retention differences among the different columns. We nevertheless offer the following analysis to demonstrate the difficulty in drawing conclusions about the separation mode, unless much more precise retention measurements are made than were apparently done[6]. First, the elution volumes for each higher molecular weight polymer sample ($\geqslant$100 000 daltons) vary by 1.3–1.6 ml. The cyano column was 30 cm long compared with 25 cm for the remaining alkyl-phase columns, which reduced this discrepancy by about $0.5/F$ ml (F = flow-rate). The flow-rate was not stated, but assuming a value of 1 ml/min, the range of elution volumes would then be 0.8–1.1 ml, or about 2% (v/v) in the good solvent. For an S value of 100, this corresponds to a difference in k' (same polymer, different columns) of about 100. This is hardly an insigificant difference.

A better test of the unimportance of the column packing surface in affecting retention is one that maintains the surface constant (same bonded phase) while changing surface area. In this case, the k' values should be identical for a given polymer sample and columns of different surface area. If a sorption retention process is involved, on the other hand, then k' should increase in proportion to column surface area. This test has been applied to the reversed-phase separation of polystyrenes, and k' has indeed been found proportional to column surface area[29] (see Fig. 4).

The recent study of Glöckner and Van den Berg[62] on the elution of various polymer samples from columns of widely differing surface area also cites similar retentions on columns of widely differing surface area. From this and other observations, they concluded that a precipitation process describes this normal-phase gradient elution system. We are inclined to agree, because of the close agreement of

retention times for the same sample and different columns and because of the large differences in column surface areas. They also noted that elution occurs at the same composition as the cloud point, which further supports their conclusion. However, it is of critical importance to determine exact compositions for both retention and cloud point measurements, inasmuch as only small differences often separate the precipitation and normal chromatograpic cases (*e.g.*, see Fig. 4 in ref. 1).

APPENDIX II

Further comments on data in Fig. 4

We can further test the data in ref. 29 for polystyrenes with molecular weights as high as 17 500 daltons (polystyrenes of greater molecular size appear to be contrained in very small pores, which affects their k' values). As the packings studied[29] were all C_{18} bonded phases, the retention per unit area of the packing surface should be constant for a given polystyrene. This means that the retention for a given solute should be proportional to the surface area of the packing, which increases as the pore diameter decreases. This relationship was shown to hold approximately[29], as measured by the apparent stationary phase volume, V_s, for each packing. That is, k' for a given solute should be proportional to V_s for the column packing, and this derived value of V_s (from values of k') should then be proportional to surface area, as measured (approximately) by the surface area of the silica particles used to prepare the column packing. We have re-examined these data in the light of the studies in ref. 55, which show that the effective surface area (value of V_s) as measured by uptake of acetonitrile from the mobile phase is reduced in small-pore C_{18} packings, presumably because of constriction of the pores due to their filling by the C_{18} phase. Revised values of the effective surface area or V_s value were reported in ref. 55 for the columns described in ref. 29. Fig. 4 shows the V_s values from ref. 29 *vs.* packing surface area, using variously the surface area of the starting silica (○) or the corrected surface area (●) from ref. 55. The resulting plot shows a good correlation between V_s and packing surface area. We conclude (for reversed-phase HPLC) that there is quantitative agreement between experimental data and a model that assumes no exclusion of large polymer molecules (17 500 daltons) from pores as small as 6 nm in diameter.

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