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Effect of sample viscosity in high-performance size-exclusion chromatography and its control

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ABSTRACT

When the sample viscosity greatly exceeds that of the mobile phase, flow instabilities occur, which lead to non-uniform flow in the radial direction. This "fingering" effect is usually greater with large-diameter than with micro-bore columns and may have worse effects in size-exclusion chromatography (SEC) than in any other chromatographic mode, as retention is shorter and dilution less important with SEC than with modes where retention is significant. This study suggests ways to reduce this viscosity effect. For example, this effect can be eliminated by controlling the concentration of a suitable mobile-phase additive which allows one to equate the viscosities of the eluent and the sample. Another possibility is to follow the sample with a plug of eluent, 0.3–0.5 column volumes wide, having a slightly higher viscosity than the sample. No spurious peaks are observed, as the plug acts as a wall prohibiting "fingering".

INTRODUCTION

Size-exclusion chromatography (SEC) is used as a preparative method that is able to separate and, thus, classify biopolymers according to their molecular size. It has the further advantages of (i) operating under very mild conditions where the biological activity of proteins can be conserved and (ii) permitting the complete elution of the entire sample with one single column volume of mobile phase [1,2]. This character would suggest applying SEC as a first fractionating step in the preparative purification of proteins. In practice, however, the amount of sample that can be applied to an SEC column is limited [3]. There are two reasons for this.

First, as for all the other retention mechanisms used in chromatography, there is an equilibrium isotherm relating the concentration of a given component in the mobile and the stationary phases, in this instance its concentration in the stagnant mobile phase inside the particles of the packing material. The equilibrium isotherm is related to the difference in Gibbs free energies of the component in the two liquid phases, a term dominated by the difference in the entropies of the solute in the bulk liquid and the liquid contained in the pores. These entropies are both functions of the

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concentration. Thus, like other modes of chromatography, SEC becomes non-linear at high concentrations.

Another phenomenon prevents or limits heavy column overloading. The viscosity of a polymer solution increases rapidly with the polymer concentration. The hydrodynamic behavior of a plug of a viscous solution moving along a packed bed in a less viscous stream is unstable. Local fluctuations of the packings permeability create perturbations of the rear of the plug which are unstable, increase exponentially and will generate fingering [4]. In such a case, the viscous plug separates into several smaller ones which propagate more or less independently. In the better case this enhances band broadening, whereas in the worse case the smaller plugs elute separately and the detector records a series of more or less well resolved bands. Although never analyzed in detail, this phenomenon has already been noticed and the difference in viscosity between the eluent and the sample bands has been blamed for excessive band broadening in attempts at performing high-concentration separations or purifications of high polymers or proteins [3,5,6]. Moore [5] reported that fingering appears when the product of the sample concentration, the sample intrinsic viscosity and the sample volume is higher than 0.05–0.10 ml.

The viscosity effect has been reported by Flodin [7]. Increasing the dextran concentration in a dilute sample of sodium chloride and hemoglobin resulted in increasingly tailing bands and in a marked decrease in the resolution. The resolution was little improved by a decrease in flow-rate. However, the effect was blamed on bed compression. Lambert [8] and Rudin [9] investigated the relationship between the retention volume and the sample concentration and attempted to derive corrections. This effect is observed at sample concentrations which are too low to promote fingering and is due to changes in the hydrodynamic volume of random polymer coils with concentration, changes caused in turn by a modification of the coil conformation. James and Ouano [10] studied the errors made in SEC measurements of the properties of polymers due to column overloading and found that their origin is in the sample viscosity. Emneus [11] avoided the band broadening effect due to fingering in the desalting of proteins by carrying out size exclusion in a batch mode. The sample is mixed with the dry packing and the highly viscous sample solution containing the proteins is separated by centrifugation from the packing containing only salt solution. Such a procedure is not chromatographic, however, and is unacceptable for difficult separations.

It has been recommended to keep the sample viscosity less than twice the mobile phase viscosity [1,12]. For proteins, it was suggested also that the sample concentration should not exceed 0.2% (w/w) or *ca.* 0.04 mg in a 20-μl sample loop for conventional analytical columns [1]. This figure is conservative and aims at avoiding other sources of difficulties also. A general method of reducing viscosity effects is to inject a larger, more dilute sample. However, there is a limit to the extent of volume overload that can be achieved this way before the resolution becomes too low for a useful purification.

As the separation mechanism is essentially entropic, it is not affected by temperature (provided that the size of the solute is not temperature dependent), whereas the sample viscosity decreases with increasing temperature. Insofar as the sample viscosity decreases faster than the solvent viscosity with increasing temperature, SEC separations are advantageously carried out near the upper temperature limit of the sample, the column packing or the equipment.

In an attempt to investigate the interplay of these high-concentration effects and to find some remedial action to improve the throughput of preparative SEC columns, we investigated the response of such a column to the injection of large volumes of high-concentration feed samples using different proteins. We also studied the influence of the viscosity of the eluent, which can be conveniently increased by adding glycerol whose small molecules have access to all the pores of the packing, but which is harmless towards the protein molecules and can easily be removed by dialysis.

EXPERIMENTAL

Columns

All chromatographic experiments were performed with a 25 cm × 4.6 mm I.D. SynChropak GPC 300 column (SynChrom, Lafayette, IN, USA) for high-performance size-exclusion chromatography of water-soluble polymers. According to the manufacturer, the average pore diameter of this material is *ca.* 300 Å and the average particle size is 5 μm. The total mobile phase hold-up volume of the column is 3.35 ml, as determined from the breakthrough time of uracil.

Chemicals

The samples used were uracil (Aldrich, Milwaukee, WI, USA), ovalbumin (from chicken egg and from turkey egg), bovine serum albumin and human hemoglobin. All proteins were purchased from Sigma (St. Louis, MO, USA). Glycerol was also obtained from Sigma.

Equipment

The chromatographic system consisted of two high-performance liquid chromatographic (HPLC) pumps (Model 302; Gilson, Middleton, WI, USA) controlled by a Gilson 621 Data Master and connected to an IBM PS/2 Model 50Z computer (IBM, Boca Raton, FL, USA). One or two six-port valves were used as sample injector or for solvent switching (Model 7510; Rheodyne, Cotati, CA, USA). Two variable-wavelength UV detectors (Spectroflow 757; ABI-Kratos, Ramsey, NJ, USA) were used; one, with a 1-mm path-length micro-cell, was set at 280 nm and the other, with an 8-mm cell, was set at 600 nm.

Detection

The elution of the protein mixture in the last experiment reported was monitored with two detectors in series, set at 280 and 600 nm, respectively. As hemoglobin absorbs in the visible range of the spectrum, it can be detected at 600 nm without interference from the colorless bovine serum albumin. At this wavelength, the absorption is low enough that a detector with a normal path length of 8 mm could be used. At 280 nm, both proteins absorb strongly (for this wavelength it was necessary to employ the micro-cell with a path length of only 1 mm). Hence it was possible to subtract the trace measured at 600 nm (multiplied by 1.3) from the one at 280 nm, isolating the contribution of serum albumin.

Viscosity measurement

For the determination of sample viscosities a Cannon-Fenske Routine Viscometer 50/286 was used (International Research Glassware, Kenilworth, NJ, USA). The kinematic viscosities of all the glycerol–water and ovalbumin–water solutions used were measured at 20°C. It was found that the viscosity of a 33% glycerol solution was 2.5 times that of water and that the dynamic viscosity of a 15% glycerol solution was approximately equal to that of a 140 mg/ml solution of ovalbumin. The viscosity of glycerol–water solutions increases less rapidly with increasing glycerol concentration than predicted by the classical logarithmic law ($\log \eta_{sol} = x_1 \log \eta_1 + x_2 \log \eta_2$, where x = mole fraction and η = dynamic viscosity), which is expected from a mixture of compounds able to be involved in many hydrogen bonds [13].

SEC experiments

The first experiments were made with water, a low-viscosity eluent (η = 1 cP at 20°C), as mobile phase. A flow-rate of 0.5 ml/min was selected. The eluent for the protein samples was phosphate buffer (pH 6.8) (0.2 mol/l in phosphate). For uracil (*ca.* 0.05 mg/ml) both the above-mentioned buffer and pure water were employed and the same results were obtained. For the experiments made using high-viscosity glycerol solutions in the aqueous buffer or large volumes of viscous samples, the flow-rate was lowered to 0.1 ml/min in order to decrease the column back-pressure and to take into account the influence of lower diffusion coefficients of the sample in the mobile phase on the column efficiency (*i.e.*, to operate the column at a nearly constant reduced velocity).

Optimization of operating procedures

Performance in chromatography is characterized by the column efficiency and the pressure required to move the mobile phase at the required flow velocity. The height equivalent to a theoretical plate, H, is given by the Knox equation [14]:

$$h = \frac{B}{v} + Av^{1/3} + Cv \tag{1}$$

where A, B and C are numerical coefficients, h is the reduced plate height ($h = H/d_p$), v the reduced velocity ($v = ud_p/D_m$), u the linear flow velocity, d_p the average packing-particle diameter and D_m the molecular diffusion coefficient of the solute in the mobile phase.

At constant mobile phase velocity, the reduced velocity is inversely proportional to the diffusion coefficient. This coefficient is related to the molecular characteristics of the solute and solvent. All correlation equations, and especially that derived by Young *et al.* [15], which gives very good results for globular proteins, show that the diffusion coefficient is inversely proportional to the eluent viscosity. Therefore, if we increase the viscosity of the mobile phase, for example by dissolving glycerol in the buffer normally used, we increase its viscosity, decrease the diffusion coefficients and increase the reduced velocity at constant mobile phase flow-rate. If we want to keep constant the reduced velocity to achieve the same column efficiency, we need to reduce the actual flow velocity [16].

Darcy's equation relates the mobile phase velocity to the column length, L, the inlet pressure, ΔP, and the specific permeability, $1/\phi$:

$$u = \frac{\Delta P d_p^2}{\phi \eta L} \tag{2}$$

Thus, at constant inlet pressure, the mobile phase velocity is also inversely proportional to the mobile phase viscosity. By keeping the inlet pressure constant when we change the mobile phase viscosity, we keep constant the reduced velocity and the column efficiency. The retention times and the cycle time increase and the production rate in preparative applications decreases in proportion to the actual mobile phase flow-rate.

RESULTS AND DISCUSSION

A series of experiments were first carried out with lysozyme and ovalbumin. As lysozyme carries a high positive charge at neutral pH, it reacts very sensitively to residual negative charges that are present on almost any silica-based stationary phase. Owing to cation-exchange interactions with the silanol groups on the silica surface, lysozyme was eluted after the dead time, with a retention factor of $k' = 0.2$ at low concentration. When the sample concentration was increased from 0.01 to 0.8 mg in the 20-μl sample injected, the retention volume of the band maximum decreased from 4.1 to 3.8 ml and the peak showed the tailing typical in overloaded elution chromatography [17]. The fact that lysozyme displays a moderate interaction with the column means that neutral molecules will show none.

The elution profile of ovalbumin displayed a different behavior (Fig. 1). When the amount injected was increased over a similar range, the position of the peak

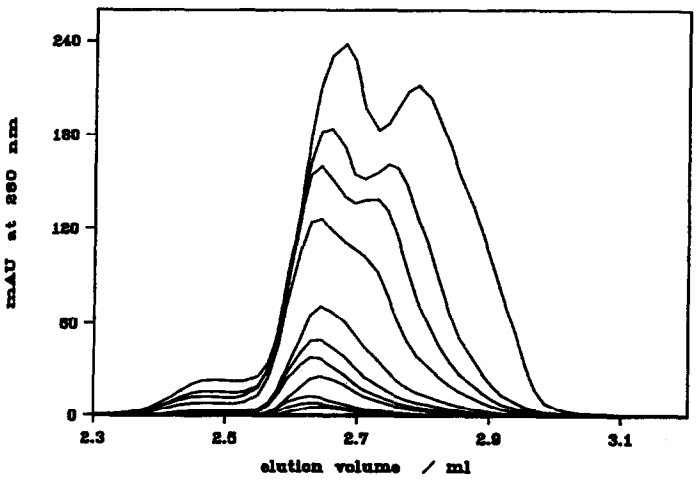

Fig. 1. Elution profiles of ovalbumin (chicken) at increasing concentrations. Sample amounts injected, 0.02–1.2 mg in 20 μl; flow-rate, 0.5 ml/min.

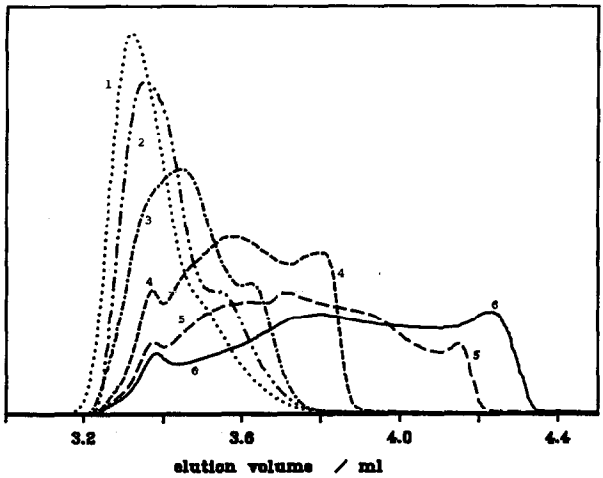

Fig. 2. Elution profiles of uracil solutions with varying glycerol content. Eluent, phosphate buffer; sample volume, 20 μl; flow-rate, 0.1 ml/min. Glycerol concentration: 1 = 0%; 2 = 10%; 3 = 17%; 4 = 33%; 5 = 50%; 6 = 67%.

maximum did not change significantly. However, a shoulder emerged towards higher retention times and grew into a second peak. This means that, although the main part of the sample molecules is eluted in the same manner as at low concentrations, an increasing fraction of the sample is retained in the column by some process. Also seen in Fig. 1 is a small shoulder preceding the main peak, but this is due to an impurity. It can be seen even at very low concentrations on the recorded chromatograms.

The following figures show an explanation for this effect, using a model system that can be manipulated more easily than a protein solution. Instead of ovalbumin solutions, we used as the sample uracil dissolved in the phosphate buffer with a variable concentration of glycerol. Uracil can be easily detected by its UV absorption, whereas the addition of glycerol allowed us flexibility in the choice of the viscosity of the sample solution within wide limits without changing the UV absorbance. As both uracil and glycerol are small molecules, the entire pore volume is accessible to them and they will not separate but will be eluted together with the total liquid volume (the total liquid volume or hold-up volume is the total volume of mobile phase in the column, contained both in the pores and between the particles of the packing).

Fig. 2 displays the chromatograms obtained when the glycerol content of the sample solution was gradually increased from 0 to 67%. The viscosities of the solutions were determined by viscosimetry. Whereas the viscosity of the most concentrated glycerol solution is about ten times that of water and therefore probably out of the range for practical samples, a solution of 33% had a relative viscosity, η/η_{H_2O}, of *ca.* 2.5. This solution was used primarily in the later experiments.

The dotted line (1) in Fig. 2 represents the chromatogram of uracil dissolved in the pure buffer. As the sample viscosity is raised by increasing the glycerol concentration (lines 2–6), an increasing fraction of the sample is spread out over up to three maxima occurring at higher retention times. In all instances, the first peak maximum remains at an elution volume of 3.35 ml, corresponding to the column total liquid

volume. This is the volume at which all the molecules that travel with the mobile phase velocity should appear. With the most viscous sample solutions, fingering of the mobile phase through the viscous sample plug takes place, the low-viscosity eluent being able to bypass part of the sample plug, leaving it behind. The effect increases with increasing viscosity of the sample plug. Only that portion of the sample that is diluted quickly will be carried with the mobile phase and arrive in the detector at the expected time. Although the effect is spectacular only at high sample viscosity, the band shape is already modified at low glycerol concentrations.

The chromatograms shown in Fig. 2 constitute an arbitrary selection from the different peak profiles found in numerous experiments. Even under identical conditions, two successive injections with the same amount of the same solution gave different chromatograms. It is not possible to reproduce the shape or exact location of the shoulders or maxima from one run to the other. This unpredictable behavior is another indication of the nature of the fingering process. Normally in chromatography, it is possible to apply the model of a "one-dimensional" column, assuming that the flow-rate and all concentrations are homogeneously distributed over the whole cross-section of the column. When fingering occurs, however, this is no longer true. In some parts of the column cross-section the mobile phase viscosity is lower and consequently the flow-rate is higher. As this is a self-amplifying process [4], the pattern for this distribution is inherently unpredictable. Consequently, this phenomenon should be relatively less important if a narrower column is used. We can also anticipate that in a large-diameter preparative column, major disturbances will take place on a scale still larger than reported in Figs. 1 and 2 [18,19]. Given the importance of the phenomenon observed, it is tempting to reverse the experiment and to inject a plug less viscous than the mobile phase.

In Fig. 3, the mobile phase contains 30% glycerol and the samples are either more or less viscous than the eluent. At the same time, the flow-rate was lowered to

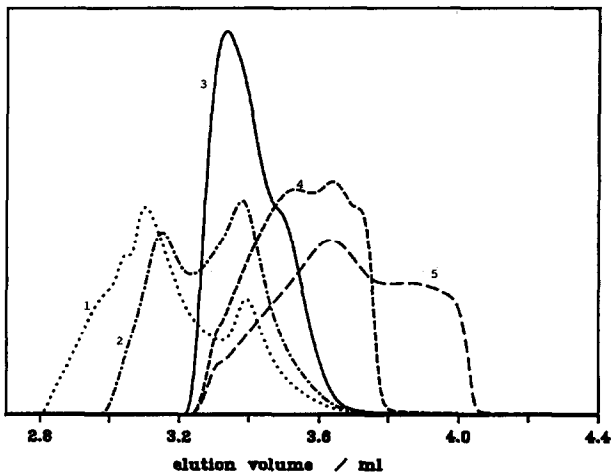

Fig. 3. Elution profiles of uracil solutions with varying glycerol content. Eluent, phosphate buffer with 33% glycerol; sample volume, 20 μl; flow-rate, 0.1 ml/min. Glycerol concentration: 1 = 0%; 2 = 17%; 3 = 33%; 4 = 50%; 5 = 67%.

0.1 ml/min, to lower the pressure drop and the reduced velocity of uracil. This more than compensates for the influence of the higher eluent viscosity, and hence for the lower diffusion coefficient of uracil in the eluent, on the column efficiency. The almost symmetrical peak in the center (3, solid line) belongs to the sample with a viscosity closest to that of the mobile phase. As the two solutions were prepared in different ways, the viscosities do not match perfectly, which leads to the appearance of a shoulder. When the viscosity increases we find elution profiles similar to those in Fig. 2.

On the other hand, when the viscosity of the sample is lower than that of the eluent, the elution band is again split into double peaks, but now this effect takes place on the band front. Part of the sample is eluted earlier than the original peak. In this instance, fingering of the sample front in the more viscous eluent occurs and it is the sample that is able to propagate along the column bed faster than the eluent. Then, the solute does not enter all the interparticle channels and does not have access to the entire packing inner porosity (see the figures in refs. 18 and 19). This leads to an effect similar to exclusion, with part of the mobile phase volume blocked by the more viscous eluent. Because of the random nature of fingering, the effect cannot be controlled and the details of the band profile are not reproducible.

The same result was obtained when a band of ovalbumin (140 mg/ml) was injected in a solution of glycerol (15%) with matching viscosity or in solutions of glycerol with higher (20%) or lower (10%) viscosities. The chromatograms obtained (not shown) exhibit a symmetrical band in the matching viscosity eluent, a bimodal band with a front eluted with a lower retention volume with the low-viscosity eluent and an important shoulder on the large retention volume side with the high viscosity eluent.

In all the experiments reported so far, the sample volume had been limited to 20 μl, the volume of the injector loop. In order to be able to vary the injection volume, a second HPLC pump was used, pumping the sample solution to the column. A switching valve determines which of the two streams would enter the column. At a flow-rate of 0.1 ml/min, it is easy to inject accurately volumes of 50 μl or larger. For Figs. 4–6, the aqueous buffer (viscosity 1 cP) or solutions at the intermediate concentration of 30% glycerol (viscosity 2.5 cP) were used as needed.

When increasing sample volumes are injected, the front of the peak does not change its position. It is always eluted at the column hold-up volume, whereas the rear of the band profile trails with increasing length (Fig. 4). We see that when we inject a sample plug more viscous than the eluent, the change from low to high viscosity still permits a stable front. However, while the sample plug moves through the column, the low-viscosity eluent following it penetrates the sample band through the formation and development of fingering. In this instance, the penetration depth is *ca.* 8 cm (Fig. 4). Only when the injection volume exceeds *ca.* 1 ml (about one third of the column dead volume) does the elution profile reach a plateau at the level of the injection concentration. In other words, it is necessary to inject a sample volume larger than 1 ml to prevent the eluent in the back of the plug from interfering with the front. As there is no retention in SEC, the front and rear boundaries should be symmetrical.

In order to separate more clearly the effects occurring at the front and at the rear of the sample band, a continuous plateau of the uracil sample solution was

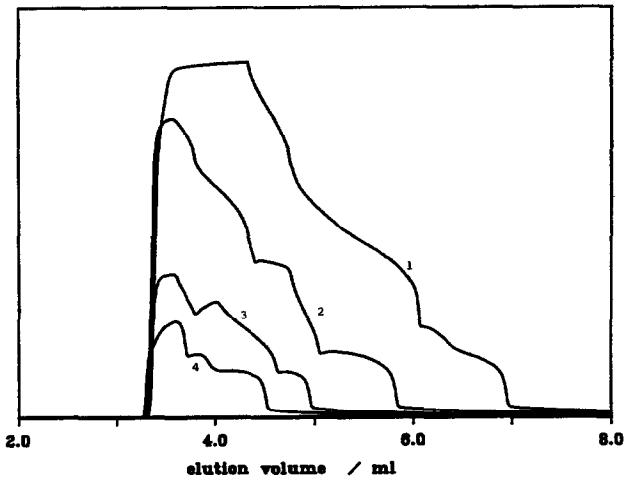

Fig. 4. Elution profiles of a viscous uracil solution, large-volume injections. Eluent, buffer; sample, 0.2–2 ml of a uracil solution containing 33% glycerol; flow-rate, 0.1 ml/min. Injection volume: 1 = 2.0; 2 = 1.0; 3 = 0.4; 4 = 0.2 ml.

delivered to the column by the second pump. Only after the plateau had reached the detector, *i.e.*, after a sample volume of 4 ml had been pumped, the valve was returned to its original position and the first pump resumed feeding pure eluent to the column. The results obtained are summarized in Fig. 5.

The bottom trace shows the plateau of a solution of uracil in pure water with water as eluent. The viscosities of both the eluent and the sample solution are equal and low. The only difference between the elution chromatogram of the plug and its injection profile is the rounding of the corners, owing to the axial dispersion in the

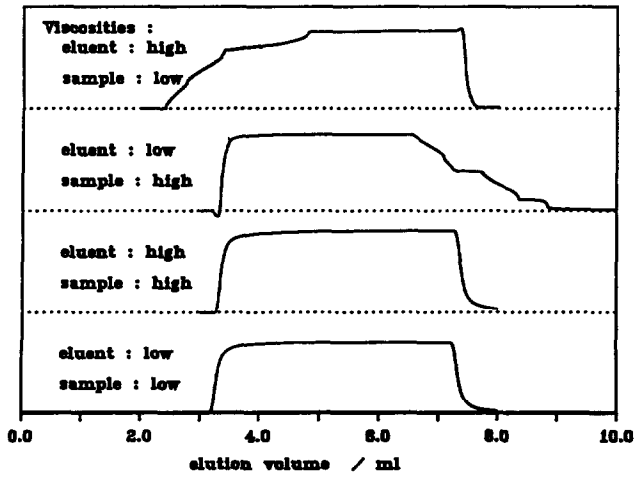

Fig. 5. Elution profiles of large rectangular plugs of uracil solutions of low or high viscosity (0 or 33% glycerol) injected into eluents of low or high viscosity. Volume of injection plug, 4 ml.

column and to the mass-transfer kinetics. When the viscosities of both sample and mobile phase solutions are high but are still equal, the second profile from the bottom in Fig. 5 is obtained. Both solutions contained 30% (v/v) of glycerol to raise their viscosity to about 2.5 times the value for pure water. As expected, this chromatogram differs from the bottom one only slightly and the differences are in the degree of band broadening. These results show that a high sample viscosity alone does not lead to any unusual effects, as long as the viscosity of the eluent and the mobile phase are equal.

The third trace from the bottom in Fig. 5 corresponds to the problematic case encountered in Figs. 1, 2 and 4. The eluent is pure water, while the uracil solution contains 30% of glycerol, as before. The front of the plateau looks almost the same as in the two lower traces. The small dip preceding the front is due to the response of the UV detector to the large change in the refractive index of the sample solution, which itself results from the high glycerol concentration added. This refractive index sensitivity of UV detectors is not unusual. It can also be seen when the sample plug contains only glycerol and no uracil. Instead of a well defined rear front, however, we find that the end of the plateau is eroded into a series of poorly defined steps. The onset of this decay occurs almost 1 ml before the expected end of the plateau, confirming our previous finding that the mobile phase which follows the viscous sample plug penetrates over such a distance (see Fig. 4).

Finally, the top chromatogram in Fig. 5 shows the fourth possible permutation of the relative viscosities of the two solutions: a sample of low viscosity is injected into an eluent of higher viscosity. Now the rear of the plateau has a well defined steep boundary. This demonstrates again that fingering arises only in the transition from a low viscosity to a higher one and not *vice versa*. As before, this step is accompanied by a small peak on the edge of the plateau, owing to the rapid change in the refractive index of the eluent. As already seen in Fig. 3 with the injections of low-viscosity, narrow-plug samples, part of the plateau moves faster than the eluent, bypassing the less accessible parts of the packed bed. The elution of the plateau begins too early, actually before the hold-up volume, illustrating again how a low-viscosity liquid can finger into a high-viscosity one.

In the practice of preparative liquid chromatography, the highly concentrated sample, for instance a protein solution, tends to be more viscous than the eluent which is normally the sample solvent. The previous figures have shown that in this instance the front of the injected plug remains stable, whereas its rear is eroded and broadened. Fingering can be prevented by raising the viscosity of the mobile phase and matching it to that of the sample. This may be inconvenient to achieve, as the viscosity of the sample must be known, and a too low or too high value will lead to unpredictably broadened peaks (see Fig. 3).

Although this procedure permits the achievement of conventionally shaped band profiles, it presents a significant drawback. Even when the viscosities are perfectly matched, the samples will experience more axial dispersion than when the column is operated at the same flow-rate but with a low-viscosity solvent. This is due to the slower diffusion rate caused by the high eluent viscosity. Increasing the solvent viscosity at constant mobile phase velocity in effect increases proportionally the reduced velocity at which the column is operated, and hence decreases its efficiency. Further, the column must be operated at an elevated back-pressure. Alternately, we

may elect to operate the column at the same reduced velocity, and hence at the same efficiency and inlet pressure as with the pure mobile phase. Then the actual flow-rate has to be lowered and the retention times and the cycle time are increased.

Another approach consists in stabilizing the rear boundary of the sample band. As the front needs no further protection, we can continue to use an eluent of low viscosity. However, the rear must be followed by a zone of high-viscosity mobile phase, for hydrodynamic stability. Immediately after the sample has been injected, it is followed by a plug of a more viscous solvent, *e.g.*, the buffered mobile phase containing an adequate amount of glycerol. This plug has to be at least as wide as the penetration depth of fingering, which is about 1 ml in our example. After the passage of an appropriate volume of viscous buffer we can return to the original eluent. It does not matter if the low-viscosity mobile phase "fingers" into the high-viscosity mobile phase plug.

Experimentally this set-up can be realized with two pumps and a switching valve that selects between the two alternating solvents and is placed upstream from the sample injector. Alternatively, one could use one pump for the main eluent and a sample injector with two loops in series, one for the sample and the other for the solvent plug. A flow scheme that permits independently loading of the two loops is through use of a ten-port valve.

Fig. 6 shows the chromatograms obtained for the injection of a 100-μl sample of a uracil solution in water containing 30% of glycerol, followed by plugs of the same viscosity with increasing volumes. For the sample alone a profile similar to those in Fig. 2 is obtained. When the sample is followed by another 100 μl of viscous eluent, the profile actually looks worse than before. Presumably, the two 100-μl bands behave as a single 200-μl injection band, which is penetrated just as easily by the following low-viscosity eluent stream. When the volume of the pusher is increased to

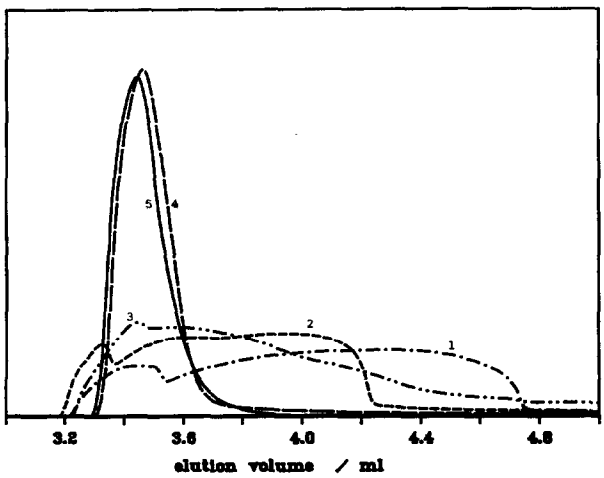

Fig. 6. Elution profiles of viscous uracil solutions. Eluent, buffer; sample, 100 μl of uracil solution containing 33% glycerol, followed by a variable volume plug of buffer with 33% glycerol. Width of viscous plug: 1 = none; 2 = 0.1; 3 = 0.4; 4 = 1.0; 5 = 2.0 ml.

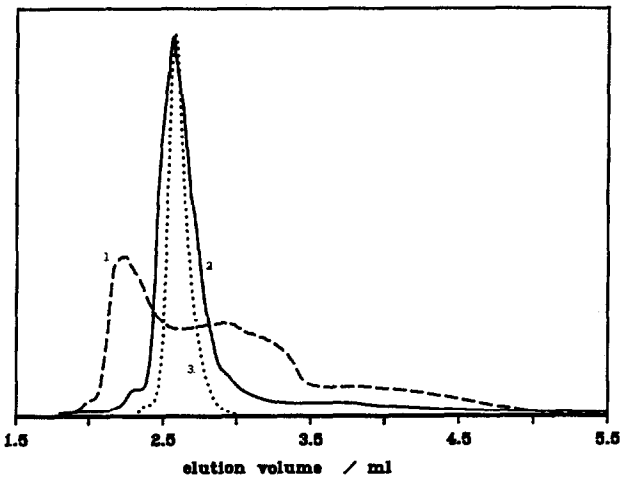

Fig. 7. Elution profiles of ovalbumin (turkey). Injection of low-concentration (expanded 15-fold, dotted line, 3), high-concentration (dashed line, 1) and high-concentration sample followed by a 1.4-ml viscous plug (solid line, 2). Concentrated sample, 2.8 mg in 20 μl; plug buffer with 15% glycerol.

400 μl, the band shape improves only slightly. However, as soon as the width of the plug reaches 1 ml, the sample peak appears narrow and almost symmetrical, very like the peak obtained for the injection of a matched viscosity sample in a high-viscosity stream of mobile phase (see Fig. 3). There is little change when the volume is increased further to 2 ml.

Now that the principle has been established with this model system, it is tested with a more realistic sample. Returning to the ovalbumin sample (*cf.*, Fig. 1), in-

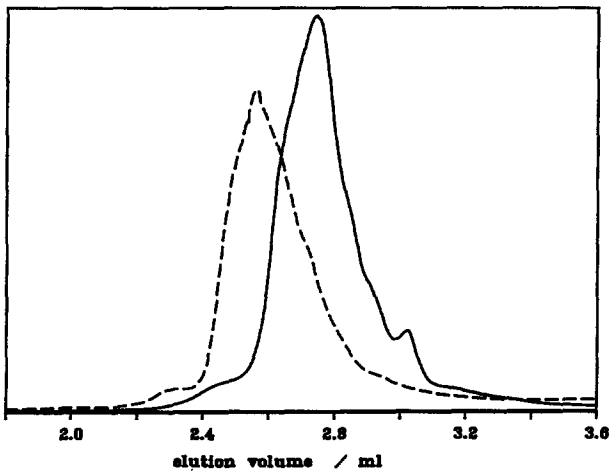

Fig. 8. Elution profiles of ovalbumin (turkey). Two ways to obtain narrow and symmetrical peaks: sample injected into eluent of matching viscosity (dashed line) or into pure buffer followed by a 2-ml plug of viscous buffer (solid line). Same sample as in Fig. 7; plug with 15% glycerol.

jections of a concentrated solution results in the elution profile shown in Fig. 7 with the dashed line (1). The first method for improving the shape of such a peak consists in matching the viscosity of the eluent with that of the sample. In this instance the sample viscosity was roughly equal to that of a 15% solution of glycerol. When chromatographed in this eluent, ovalbumin produced an almost symmetrical peak (solid line, 2). It is compared with the peak profile obtained at low concentration that has been expanded by a factor of 15, to the same peak height (dotted line, 3). The two peaks show the same symmetry, but the chromatogram taken with the high-viscosity eluent at the same mobile phase flow-rate displays significantly higher band broadening. The high-concentration band is almost twice as wide as the low-concentration band and the column efficiency is lower.

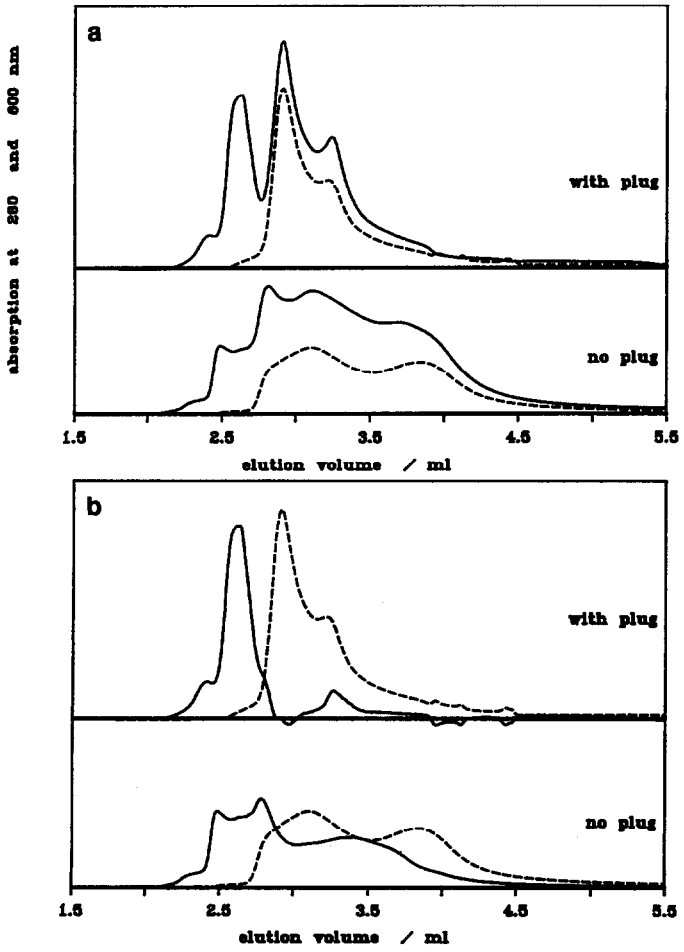

Fig. 9. Elution profiles of a mixture of bovine serum albumin and hemoglobin. Eluent, buffer; sample, 1.4 mg of each protein in 20 μl. Above: injection followed by 2-ml plug of buffer with 15% glycerol. Dashed lines, detector signal at 600 nm; solid lines, (a) signal at 280 nm and (b) difference between signals at 280 and 600 nm.

The result of the alternative procedure of following the injection of the sample by the injection of a viscous plug is shown in Fig. 8. When the protein sample band is followed by a 2-ml plug of the 15% glycerol solution, the peak shape obtained is equivalent or even narrower than that produced with an eluent of matching viscosity. In the experiments with uracil the sample band moved with the same velocity as the following plug, as both solutions contained only small molecules which have access to the entire inner porosity of the packing. Now, with the protein solution, the sample is partially excluded from the pore volume and leaves the viscous plug behind. Even though the protein and the glycerol molecules do not travel together any more, the front of the plug still has a stabilizing effect on the sample band.

Fig. 9 demonstrates the viscosity effect on a size-exclusion separation of the two proteins bovine serum albumin and hemoglobin and the improvement brought about by efficient control of this effect. For this study, two detectors were used; one set at 280 nm responds to both proteins and the other set at 600 nm responds only to hemoglobin. The chromatograms in Fig. 9a show the two detector signals. The chromatograms in Fig. 9b show the individual profiles of the two proteins. After calibration to determine the ratio of the detector responses for hemoglobin, the profile for bovine serum albumin is obtained by subtraction of the signal of the second detector from the signal of the first (in Fig. 9a). This procedure results in the chromatogram in Fig. 9b, containing the individual elution profiles of the two proteins.

The lower chromatograms in Fig. 9a and b show the elution profile obtained normally by injecting 20 μl of the concentrated protein in solution into a pure phosphate buffer. In the upper chromatogram, the injection was followed by a 2-ml plug of buffer containing 15% of glycerol. It is clear that for the lower chromatogram the separation is not sufficient. In contrast, when the injection was followed by a viscous plug (upper chromatograms), a satisfactory separation of the two protein components was achieved. The positive influence on the separation of the injection of a viscous plug after the sample is obvious.

CONCLUSIONS

When operating with high-viscosity samples, the least detrimental approach seems to be to follow the sample with a plug of mobile phase having a viscosity equal to or slightly higher than that of the sample. Glycerol appears to be a good mobile phase additive for this purpose, although low-molecular-weight carbohydrates (*e.g.*, sugar) could also be used. The band profile obtained is nearly identical with that of a very small plug. As the most important sample component moves faster than the unretained glycerol, the sample propagates in a low-viscosity mobile phase. Therefore, it is not necessary to decrease the mobile phase flow-rate in order to keep constant the reduced velocity of the main sample component as it would be if a high-viscosity eluent is used as the mobile phase.

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