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CONCENTRATION AND TEMPERATURE EFFECTS IN GEL PERMEATION CHROMATOGRAPHY

S. R. HOLDING*, G. VLACHOGIANNIS and P. E. BARKER*
Chemical Engineering Department, University of Aston, Birmingham (Great Britain)
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SUMMARY

Significant changes in the fractionation of dextran have been observed when conducting semi-continuous gel permeation chromatographic experiments using feeds of different dextran concentration. Using a batch analytical chromatographic apparatus with a column packed with Porasil C and labelled dextrans FITC dextran 20, FITC dextran 40 and FITC dextran 70, concentration and temperature effects on gel permeation chromatography have been determined. Background concentrations of up to 200 g/l and temperatures between 20–80°C were studied.

INTRODUCTION

Dextran is a polymer of glucose used clinically as a blood plasma expander and as a carrier for iron in the treatment of anaemia[1]. We have been developing methods for the chromatographic refining of dextran for some time[2–4]. This refining of dextran is achieved by semi-continuous gel permeation chromatography (GPC) on an apparatus consisting of ten columns (70 cm × 5.1 cm I.D.) packed with porous silica beads (200–500 μm). The original semi-continuous chromatograph used in this work has already been described in detal[4,5]. Since then the chromatograph has been improved by the introduction of stainless-steel columns, higher column efficiencies and a heated air-bath. The use of stainless-steel columns allows the use of higher pressures (16 bar), which has enabled us to achieve the successful fractionation of dextran at 600 g/h at ambient temperature. The use of an increased temperature (80°C) with a consequential decrease in viscosity should permit us to attain higher throughputs.

There are many theories to explain the mechanism of GPC, all based on conditions for which it may be assumed that there is no chemical interaction between the solutes being separated and the chromatographic packing. Separation is achieved by the ability of the packing to retard molecules according to size.

In most forms of liquid chromatography the composition of the mobile phase

* Present address: PSCC, RAPRA, Shawbury, Shrewsbury, Great Britain.

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is critical, whereas in GPC the nature of the mobile phase has little effect, only determining the hydrodynamic volume of the solutes and their diffusivities.

One of the initial observations from our work on the semi-continuous GPC of dextran was that the dextran feed concentration had a dramatic effect on the chromatography. In two early experiments, it was found that by increasing the concentration of the feed from 1 to 20% changed the fractionation from removing the lowest 10% molecular weight material to removing the highest 10% molecular weight material[5,6]. The marked change in chromatographic behaviour was confirmed in a separate experiment by fractionating labelled dextran with eluents containing various concentrations of dextran[5,6]. The labelled dextran was found to elute later, with a broader peak, at higher background concentrations of dextran.

Changes in elution volume have been observed with changes in sample concentration[7–9]. These observed elution volume changes have usually been attributed to changes in the hydrodynamic volume of the solutes, the higher polymer concentrations causing shrinkage of the solvated molecules. Large changes in the elution volume of proteins have been observed in GPC on Sephadex xerogels when polymer is added to the eluent[10–12]. Unfortunately, the interpretation of the chromatographic behaviour is difficult owing to the shrinkage of the xerogel packings under these conditions. More recently, work has been carried out on rigid aerogels with polymer present as a background in the eluent[13,14], but in these instances only low concentrations of background polymer have been employed.

Analytical GPC at increased temperature is usually used only when required by the low solubility of the polymer or the high viscosity of the polymer[15]. There has been some recognition that GPC at higher temperatures will improve the efficiency and resolution[16], but most analytical GPC is still carried out at ambient temperature.

In the semi-continuous chromatographic refining of dextran it will be economically desirable to maximize throughput by the use of high feed concentrations, with consequentially a high background concentration of dextran, which will require increased temperatures to reduce the viscosity and hence the pressure drop across the refiner.

This work was aimed at obtaining a fuller understanding of the effect of concentration and temperature on the fractionation of dextran by GPC, which would then allow us to be more predictive in the operation of our semi-continuous chromatographic refiner.

EXPERIMENTAL

A simple batch analytical chromatographic apparatus was used. The eluent was pumped with a positive displacement pump (Series II, Metering Pumps, London, Great Britain). The samples were introduced with a loop injection device (Spectroscopic Accessory Co., Sidcup, Great Britain) fitted with a 100-μl sample valve. For most of the work an ultraviolet deterctor was used (Altex, Berkeley, CA, U.S.A., Model 153), although a differential refractometer (Laboratory, Data Control, Stone, Great Britain, Model 1107L) was used to compare the chromatographic behaviour of labelled and unlabelled dextrans.

The chromatographic column was a 1 m × 4 mm I.D. stainless-steel tube fitted

with meshes and Swagelock fittings (all obtained from HETP, Macclesfield, Great Britain). The column was filled with Porasil C, 30–75 μm diameter (Waters Assoc., Stockport, Great Britain) and the temperature of the column was controlled with a circulator [Grant Instruments (Cambridge) Ltd., Cambridge, Great Britain, Model FH15] pumping water through a glass jacket.

The labelled dextrans used in this work (Table I) were FITC dextran 20, FITC dextran 40 and FITC dextran 70 (Pharmacia, Uppsala, Sweden). These are narrow-molecular-weight fractions labelled with fluorescein isothiocyanate with a very low degree of substitution (less than 0.01 mole per mole)[17]. Hydrolysed Procion Red Dye (ICI, Macclesfield, Great Britain) was found to behave as a totally included solute.

TABLE I

MOLECULAR WEIGHTS OF DEXTRANS USED

Material	$M_{50\%}$*	$\bar{M}_w$	$\bar{M}_n$	$\frac{\bar{M}_w}{\bar{M}_n}$
FITC Dextran 20	18,000	19,000	17,500	1.09
FITC Dextran 40	32,500	39,000	31,000	1.26
FITC Dextran 70	61,000	67,000	52,000	1.29
Dextran BT216a	26,214	30,524	21,004	1.45

* $M_{50\%}$ is the molecular weight corresponding to 50% of the area of the chromatogram.

Earlier work has shown that all the labelled dextrans behaved as excluded solutes when chromatographed on columns packed with porous silica unless the ionic strength of the eluent was increased; therefore, sodium chloride was added to each eluent solution to suppress the ionic exclusion effects. Sodium azide was also added to inhibit bacterial growth. The composition of the three eluent solutions were as follows:

(a) 0.5% sodium chloride and 0.02% sodium azide, pH = 6.59;

(b) 10% dextran 40 (BT 216a), 0.5% sodium chloride and 0.02% sodium azide, pH = 6.2;

(c) 20% dextran 40 (BT 216a), 0.5% sodium chloride and 0.02% sodium azide, pH = 5.97.

The dextran 40 (BT 216a) (weight-average molecular weight *ca.* 40,000) was obtained from Fisons (Pharmaceutical Division). The dextran 40 concentration in the sample solutions was adjusted, where possible, to give a total concentration equivalent to that of the eluent. The eluent flow-rate was maintained at *ca.* 0.4 cm³/min for all three eluent solutions. The accurate flow-rates were determined by collecting and weighing the eluate for each chromatography, a correction for density being made where necessary.

The efficiencies for each eluent/temperature combination was measured from the elution of hydrolysed Procion Red Dye, using the equation

$$N = 8\left(\frac{t_R}{W_{h/e}}\right)^2 \qquad (1)$$

where N is the number of theoretical plates, t_R is the peak retention time and $W_{h/e}$ is the peak width at peak height, h, divided by e, the base of the natural logarithm.

The area of each dextran chromatogram was calculated using Simpsons rule and the 50% area elution volume calculated and recorded.

RESULTS

The results for each eluent composition are summarized in Tables II–IV.

The effect of increasing the dextran background concentration can be seen in Fig. 1. Compared with the eluent containing no dextran, 10% dextran in the eluent causes an average increase in elution volume for the three solutes of 12%, whilst 20% dextran causes an approximately 19% increase in elution volume.

Hydrolysed dye, used as a totally included solute, exhibited an increase in elution volume of less than 4%, due to the increase in concentration, as can be seen marked on the baselines in Fig. 1. Considerable tailing was produced by the high background concentrations of dextran.

TABLE II

ELUTION VOLUMES (cm^3) AND EFFICIENCY OF THE CHROMATOGRAPHIC SYSTEM USING THE DYE AND THE FITC DEXTRANS WITH ELUENT (a): 0.5% NaCl AND 0.02% NaN_3

Solute	*Temperature (°C)*			
	Ambient	*40*	*60*	*80*
1% FITC dextran 70	12.90	12.36	12.07	11.32
1% FITC dextran 40	13.04	12.74	12.76	12.65
1% FITC dextran 20	14.10	13.82	13.72	13.68
0.1% dye	14.97	14.89	14.74	14.64
Efficiency (plates) of the system measured by the dye	428	503	634	738

TABLE III

ELUTION VOLUMES (cm^3) AND EFFICIENCY OF THE CHROMATOGRAPHIC SYSTEM USING THE DYE AND THE FITC DEXTRANS WITH ELUENT (b): 10% DEXTRAN 40, 0.5% NaCl AND 0.02% NaN_3

Solute	*Temperature (°C)*			
	Ambient	*40*	*60*	*80*
1% FITC dextran 70	14.69 (cm^3)	14.06	13.88	13.79
1% FITC dextran 40	15.23	14.87	14.62	14.30
1% FITC dextran 20	15.09	15.21	15.27	14.83
0.1% dye	15.24	15.25	15.29	15.09
Efficiency (plates) of the system measured by the dye	878	806	937	1059

TABLE IV

ELUTION VOLUME (cm^3) AND EFFICIENCY OF THE CHROMATOGRAPHIC SYSTEM USING THE DYE AND THE FITC DEXTRANS WITH ELUENT (c); 20% DEXTRAN 40, 0.5% NaCl AND 0.02% NaN_3

Solute	*Temperature (°C)*			
	Ambient	*40*	*60*	*80*
1% FITC dextran 70	15.03 (cm^3)	14.86	14.54	14.28
1% FITC dextran 40	15.76	15.58	15.52	15.18
1% FITC dextran 20	15.99	15.87	15.59	15.62
0.1% dye	15.51	15.53	15.46	15.24
Efficiency (plates) of the system measured by the dye	526	749	814	886

In Fig. 2 the results at ambient temperature are presented in another form, the elution volume being plotted against the background concentration for each FITC dextran fraction and the dye. It can be seen that the change in elution volume is a linear function of concentration for FITC dextran 20 and the dye, but a log-linear function for the higher-molecular-weight dextrans FITC dextran 40 and 70. The concentration effect on the elution volumes of dextran at higher temperature follows a similar profile to those at ambient temperature and hence these graphs have not been included. The results are similar to those obtained by Boni *et al.*[18,19] and Lambert[20].

An increase in temperature usually produces a decrease in elution volume. The general effects are most obvious when comparing the extremes of ambient temperature and 80°C (Fig. 1). The FITC dextran 70 is the most affected and FITC dextran 20 the least affected by the temperature change. Also, the effect of temperature is more pronounced in the absence of background dextran in the eluent.

DISCUSSION

Concentration effects, *i.e.*, the dependence of the elution volume and of the width of the elution curve on concentration and overall amount of injected polymer solution in GPC, have been observed by many workers. This change in elution volume due to a change in the concentration has usually been ascribed to three effects: viscosity phenomena in the interstitial volume; the change in the effective size of permeating molecules and thus a change in the distribution coefficient according to the respective calibration graph; and secondary exclusion. The first two effects lead to an increase of elution volume, whereas the last causes a reduction in elution volume with increasing concentration.

Janča, particularly, has implied that a significant proportion of the concentration effect is due to viscosity[21–23]. However, Basedow *et al.*[24] have recently shown that the separation of dextran on a rigid chromatographic packing is an equilibrium process, not affected by increased viscosity.

It has been assumed that chromatography with polymer in the eluent may be described as a two-polymer phase partition[11]. It has also been suggested that the

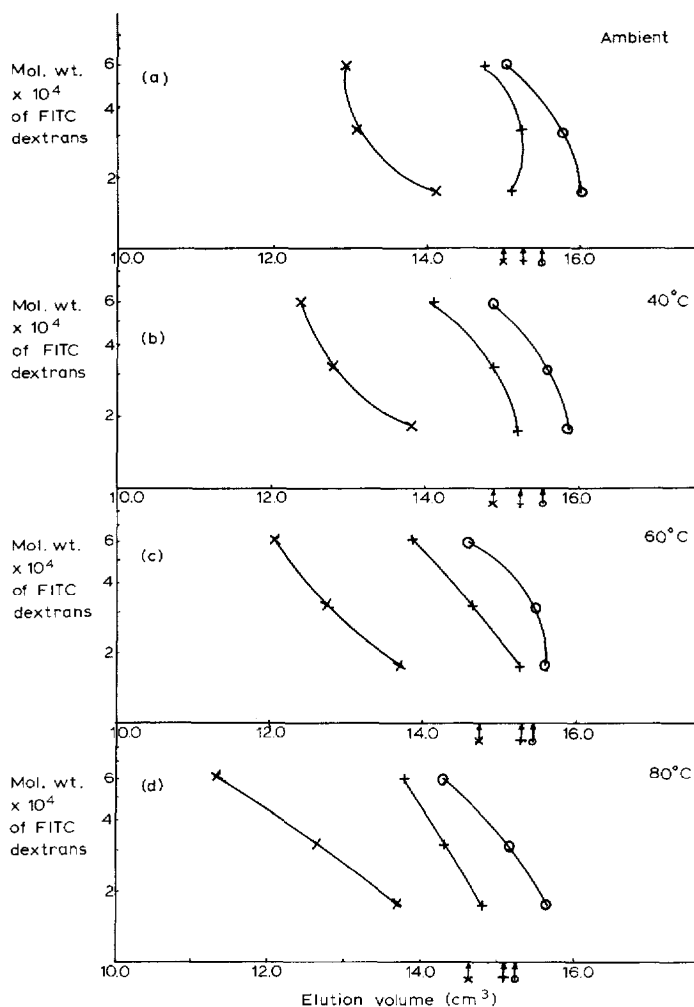

Fig. 1. Change in elution volume of labelled dextrans with increasing background dextran concentration (×, zero concentration; +, 100 g l^{-1}; O, 200 g l^{-1}) and increasing temperature: (a) ambient; (b) 40°C; (c) 60°C; (d) 80°C. The arrows along the abscissa indicate the elution volume of marker dye at the various dextran concentrations.

mobile phase polymer can act as an additional chromatographic packing[10,25]. Laurent[26,27] has clearly demonstrated that concentrated dextran solutions do display exclusion properties. The fact that an increased polymer concentration in the sample causes a decrease in the hydrodynamic volumes of the polymer molecules is frequently used to explain increased elution volumes, and this effect is consistent with theoretical predictions[28,29], although complications with different molecular weights behaving differently has been observed[14].

We have used background polymer concentrations which are much higher than those used by most other workers and the observation that solutes can elute at a volume greater than the total column eluent volume does not allow a simple explanation based only on changes in hydrodynamic volume.

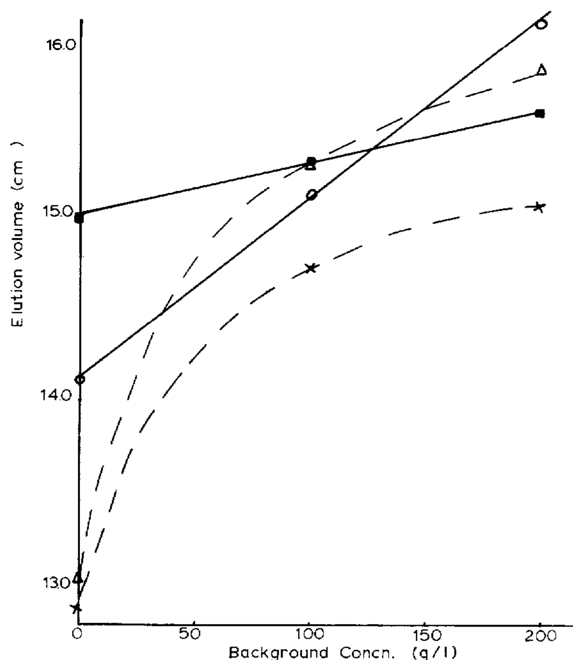

Fig. 2. Effect of background concentration on the elution volume of a GPC elution curve (at ambient temperature). □, Dye; ○, FITC dextran 20; △, FITC dextran 40; ×, FITC dextran 70.

Janča[30] suggested that because at higher concentrations the pore and void volumes available to a molecule in a column are smaller than at dilute conditions, owing to the higher fraction of liquid volume in the column occupied by the solute molecules, secondary exclusion leads to a reduction in the elution volume of a molecule with an increase in column concentration. However, our work showed that elution volume increases as the column concentration increases and hence this effect probably is not as significant as the other effects that lead to an increase of elution volume with increase in concentration.

Another explanation that may lead to distribution coefficients greater than unity is that dextran molecules at higher concentration are linked together, so that it is difficult for them to move down the column, through the void volumes between the gel particles. Although this explanation seems unlikely, it can explain why the dye at higher concentrations is eluted before dextran.

The possibility that the polymer in the eluent may behave as a chromatographic packing, with its own exclusion properties, leads to some surprising observations. A 20% (w/v) solution of dextran is similar to a cross-linked dextran with a water regain of 5 cm^3/g. In our case the water regain is limited by the water available rather than the cross-linking. The dextran used in the background will be able to permeate many of the packing pores; therefore, the volume available to the background dextran will be greater than just the interstitial volume and a 20% (w/v) dextran solution may be expected to be a 10% (w/v) solution when "on-column". Hence the background dextran would be expected to behave as a packing with a water regain between 5 and 10 cm^3/g (*i.e.*, Sephadex G-25 and G-50)[31]. This represents a GPC

packing of relatively low exclusion limit (between 5000 and 10,000 daltons, for dextran). In fact, most of the dextran used in the eluent would be excluded from a gel with such a low exclusion limit.

Similarly, the 10% (w/v) dextran solution may be expected to behave like a cross-linked dextran with a water regain between 10 and 20 cm^3/g (*i.e.*, Sephadex G-50 and G-100). The exclusion limit of such a gel would allow most of the dextran to permeate its pores to some extent. In contrast, the rigid Porasil C packing has a pore size range which would allow most of the background dextran to permeate its pores.

These observations are consistent with elution volumes greater than the total solvent volume of the column, implying that the solutes are spending a greater proportion of time in the rigid column packing pores than in the interstitial volume because the "secondary gel" has a lower exclusion limit in the interstitial volume than the pore volume due to the higher polymer concentration in the interstitial volume. It is important to appreciate that the labelled dextran is only there to help identify how the background dextran is behaving. A very small molecule should be totally permeable through both the rigid packing pores and the less well defined "gel" in the interstitial volume. The dye, used as a small solute, apparently experiences the same effect but to a much lesser extent.

The shapes of the "calibration" graphs in Fig. 1 are surprising, but the consistency between the various temperatures is reassuring. It should be remembered that those chromatographic runs with no polymer in the eluent will not be independent of concentration. The large sample size and moderate sample concentration will produce the effects observed by Janča and other workers[13,21,22,25]. The changing shape of the "calibration graphs" may be due to the different solvation effects exhibited by the different molecular weight species[14]. The low-molecular-weight species are preferentially solvated, whereas the high-molecular-weight molecules are less solvated and hence smaller than would be expected from their behaviour in dilute solutions.

The various concentrations and molecular weights of dextran in the different sized pores present a very complex situation. The low-molecular-weight dextran can permeate most of the pores and is particularly subject to many environments, a situation which probably contributes a lot to the tailing observed at the higher dextran concentrations.

The influence of temperature appears to be less complex. It seems reasonable to suppose that the hydrodynamic volume will increase significantly with increased temperature. This increased hydrodynamic volume will cause a reduction in elution volume, and this is evident when there is no dextran background concentration. The higher molecular weights appear to exhibit the greatest effect. When there is dextran present in the eluent, the effect of increasing the temperature is much less marked. Probably the increase in hydrodynamic volume is limited by competition for solvent.

The efficiency of the chromatographic column was improved by increasing the temperature. This is probably due to a decrease in the viscosity of the eluent and increased diffusion coefficients for the dextran.

CONCLUSION

The presence of a high concentration of polymer in the eluent has a dramatic effect on the elution volume of polymeric solutes. It is suggested that the predominant cause is the background polymer behaving as another size exclusion material. This behaviour is further complicated by a non-uniform decrease in hydrodynamic volumes of solutes depending on their molecular weight and several other effects.

An increase in temperature improves the efficiency in GPC and causes a decrease in elution volume owing to increased hydrodynamic volumes, but this effect tends to be "swamped" by the presence of background polymer in the eluent.

In our large-scale semi-continuous chromatographic refiner, the dextran concentration and molecular weight distribution vary throughout the different sections of the apparatus, but these parameters can be determined. This work should allow us to predict the chromatographic behaviour of any dextran molecular weight species in any section and eventually build up a model describing the operation of the refiner.

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SYMBOLS

e	constant equal to 2.718;
h	height of chromatogram;
$\bar{M}_n$	number average molecular weight;
$\bar{M}_w$	weight average molecular weight;
$M_{50\%}$	molecular weight at 50% area of the chromatogram;
N	number of theoretical plates;
t_R	elution (retention) time;
V	pore volume;
V_0	interstitial (void) volume;
V_R	elution (retention) volume;
W	width of chromatogram.

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