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CHARACTERISTICS OF A NEW AGAROSE MEDIUM FOR HIGH-PERFORMANCE GEL FILTRATION CHROMATOGRAPHY

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SUMMARY

Some physical and chromatographic properties of Superose 6B®, a new agarose-based material for medium speed high-performance gel filtration, have been investigated. The chromatographic particle size was derived from the volume-size distribution, and the apparent particle diameters of the tested batches were between 32 and 35 μm. The material was easy to pack with standard laboratory equipment and yielded efficient columns with a reduced plate height of two for a totally permeating compound. The high permeability of the matrix ($V_i/V_0 \approx 2.3$) was advantageous for both high resolution and high sample loads. The molar mass separation range was 10^3–$4 \cdot 10^6$ for proteins and 10^3–10^6 for dextrans. The semi-rigid material was not substantially compressed at linear flow-rates below 40 cm/h where the Blake–Kozeny equation was found to be valid. Column efficiency was strongly dependent upon sample molecular weight, and in order to compensate for the slow mass transfer of macromolecules the columns were eluted at 10–20 cm/h resulting in separation times of 3–5 h. However, faster analyses are possible for samples eluting in the middle of the separation range.

INTRODUCTION

During the last five years several new media for aqueous permeation chromatography, *i.e.*, gel filtration, have been introduced[1,2]. Attention has focused upon the production of small beads that when packed in a column withstand pressure drops of greater than 10 MPa. This is made possible by a substantial increase in matrix volume as compared to traditional soft gel media[3]. This leads to a reduction in separation volume, which partly reduces the positive effect on resolution of the smaller bead size[3], and sorptive effects which have been noted for several of the materials[4–12].

The gain in pressure resistance of the materials is used to increase the linear flow-rate of the eluent and thus decrease the time of analysis. However, the separation efficiency is primarily governed by bead size, separation volume, porosity curve, packing efficiency, linear flow-rate and solute diffusion coefficient. As has been experimentally verified, it is not always possible to utilize a high flow-rate because the

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actual sample diffusion in the bead size used may be a limiting factor, leading to poor resolution[13]. This is especially true for high-molecular-weight proteins, even when utilizing small bead-size material[14].

It is well known that agarose-based matrices are very suitable for gel filtration[15]. Until now, however, the commercially available agarose media have required low linear flow-rates for high resolution due to the large bead sizes of the materials. This paper describes the basic properties and some applications of a new cross-linked agarose medium with a bead size of 30 μm for use in medium speed high-performance gel filtration chromatography.

EXPERIMENTAL

Bead size distribution

The bead size distribution of Superose 6B was determined by projecting a microscope image of the beads onto a digitizer table (Kontron) with the aid of a Zeiss microscope, equipped with a projection tube. The diameters of these beads were enlarged 1000 times, then digitized with a stylus and the data automatically transferred to a desk computer (HP 9835, 120 kB RAM). After counting a sufficient number of particles, *i.e.*, *ca.* 1000, the particle size distribution was calculated and a report generated on a printer/plotter (HP 7245A). Software for data communication, calculations and evaluations was developed in our laboratory[16].

Matrix rigidity

The enhanced rigidity of the cross-linked beads was checked by measuring the pressure drop over a column, when running a linear flow gradient. The column was packed by pouring a dilute (*ca.* 50%, v/v) gel slurry into a 10 × 500 mm glass column, *i.e.*, an extended HR 10/10 column, equipped with an extension glass tube (Superloop 50 cm^3) and then pumping distilled water through the column at a flow-rate of 40 cm^3/h with a syringe pump (P-500, Pharmacia Fine Chemicals, Uppsala, Sweden). After *ca.* 2 h the column was packed and the inlet adaptor fixed. The pump was then connected to a high pressure pump (Constametric III, LDC) which was controlled by a gradient programmer (Gradient Master, LDC) to run a linear flow gradient from 0 to 2 cm^3/min in 3 h. The analog signal from a pressure sensor (spare part No. 19-4331-01, Pharmacia) was fed to a recorder (REC-481, Pharmacia) and the back pressure was related to the flow-rate with the aid of the chart speed. The theoretical pressure drop was calculated with the Blake–Kozeny equation as adapted by Ladisch and Tsao[17]

$$\frac{\Delta p}{q} = \frac{600\,\mu L}{\pi D_{mp}^2 D_c^2} \cdot \frac{(1-\varepsilon)^2}{\varepsilon^3} \approx 3.18 \times 10^{-6} \cdot \frac{L}{D_{mp}^2 D_c^2} \cdot \frac{(1-\varepsilon)^2}{\varepsilon^3} \quad (1)$$

where Δp is the pressure drop in kPa, q is the flow-rate in cm^3/min, μ is the viscosity (1.67×10^{-5} min · N/m^2, water, 20°C), L is the bed height in cm, D_{mp} is the mean effective particle diameter in cm, D_c is the column diameter in cm and ε is the void fraction, *i.e.*, the ratio of chromatographic void volume to the geometric bed volume. The void volume was calculated from the first inflexion point on the chromatogram of Blue Dextran 2000.

Chromatographic performance

The columns used in the chromatographic experiments were packed according to the following procedure. The column (K 16/70) was mounted vertically and equipped with a packing reservoir (R 15/16). The air trapped under the net in the bottom end piece was then removed by injecting 2 ml of buffer through the outlet tubing with the aid of a syringe. The contents of a bottle of Superose 6B (125 cm^3) were homogenized by vigorously shaking the slurry. The slurry was poured into a beaker and an equal amount of the buffer (0.05 *M* phosphate pH 7.0) was added. The dilute slurry was then poured into the column, the remaining volume of the reservoir filled with buffer and a lid attached and tightened. A peristaltic pump (P1, 3.1 mm I.D. tubing) was attached to the reservoir inlet and the column packed at a nominal flow-rate of 300 cm^3/h and a constant pressure of 0.1 MPa. The flow-rate gradually decreased to approximately 50 cm^3/h as the back pressure increased during the packing procedure. The packing was completed after 5 h and the reservoir was then dismantled and an adaptor (A16), equipped with a new net, was inserted into the column. The gel bed was stabilized by running the column at 50 cm^3/h for 30 min and thereafter the adaptor was finally fixed.

The presence of anionic groups on the matrix was examined by measuring the adsorption of a basic protein on a freshly prepared column. Four milligrams of cytochrome *c* (Table I, isoelectric point 9.0) were eluted with the 0.05 *M* phosphate

TABLE I

DATA FOR PROBE MOLECULES AND TYPICAL YIELDS ON SUPEROSE 6B

Sources: A = Pharmacia Fine Chemicals (Uppsala, Sweden); B = Kabi (Stockholm, Sweden); C = Sigma (St. Louis, MO, U.S.A.); D = Serva (Heidelberg, F.R.G.); E = Merck (Darmstadt, F.R.G.); F = University Hospital of Uppsala, Uppsala, Sweden.

Probe	*Source*	*Lot No.*	*Molecular weight (monomer)**	*Yield (%)*
Blue Dextran 2000	A	C 173	*ca.* $2 \cdot 10^6$	97
Thyroglobulin	A	C 047	669,000	91
Ferritin	A	C 171	440,000	93
Catalase	A	C 063	232,000	97
Immunoglobulin G	B	1064	160,000	
Aldolase	A	C 060	158,000	94
Bovine serum albumin (BSA)	A	C 166	67,000	96
Ovalbumin	A	C 168	43,000	101
β-Lactoglobulin	C	F8040	35,000	
Chymotrypsinogen A	A	C 165	25,000	103
Myoglobin	C	F7036	18,800	
Lysozyme	C	F8080	14,400	99
Ribonuclease A	A	C 167	13,700	96
Cytochrome *c*	C	F7050	12,400	98
Bacitracin	D	I	1450	
Cytidine	E		243	
AMP	C	A2002	347	
Serum (pooled)	F			

* Data from the manufacturer.

buffer at pH 7.0. The adsorption was determined by comparing the absorbance at 280 nm of the collected effluent to that of a diluted sample solution.

The calibration curve for proteins was determined by injecting 500 μl of the sample (see Table I) with a valve (V-7) and eluting with buffer (0.05 *M* phosphate in 0.15 *M* sodium chloride, pH 7.0) at a flow-rate of 20 cm^3/h with the aid of a high-precision pump (P-500). The absorbance of the effluent was continuously monitored (UV-1, HR 10) at 280 nm and the detector signal traced with a recorder (REC 481). The chromatogram for cytidine was used for the calculation of the maximum column efficiency from the elution volume and the peak width at half peak height.

The calibration curve for dextrans was obtained from the elution of dextran fractions (Dextran T 10, T 20, T 40, T 70, T 110, T 150 and T 250, Pharmacia)[18]. The effluent was continuously monitored by an interferometer (Multiref 902, Tecator) as previously described[19].

Unless explicitly stated, all equipment used was from Pharmacia. Dextran T 20, T 110, T 150 and T 250 are however no longer available from Pharmacia.

RESULTS AND DISCUSSION

Bead size distribution

The particle size distribution of Superose 6B lot No. 28724 is shown in Fig. 1. Various estimates of this distribution, such as mode, mean and median, are used to assign a value to the bead size. The corresponding estimates may also be derived from the volume distribution curve, *i.e.*, a plot of the relative volume of particles with a certain diameter *versus* the particle diameter. This distribution is more influenced by the appearance of large particles than the former number distribution. Particle size estimates for two lots of Superose 6B are given in Table II and it is seen from the variation in these estimates that it is important to refer to the calculation

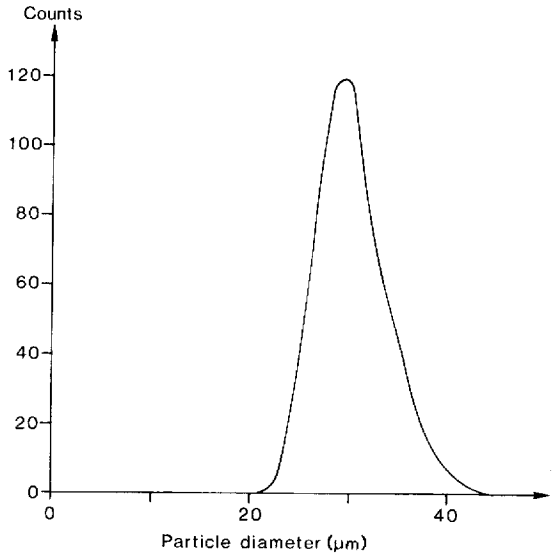

Fig. 1. Particle size distribution of Superose 6B (lot 28724).

TABLE II

PARTICLE SIZE ESTIMATES FOR SUPEROSE 6B

In the formulae N is the total number of particles (Σn_i), V the total "volume" of particles ($\Sigma n_i \times d_i^3$), n_i the number of particles and v_i the volume fraction ($\Sigma n_i \times d_i^3 \times V^{-1}$) of particles with diameter d_i and c is the ratio of the standard deviation and the mean, $\bar{D}_p$, of a Gaussian number distribution.

Estimate		*Value*	*(μm)*	*Formula*	*Ref.*
Number distribution,	mode	30.0	31.7	d_i for max(n_i)	
	mean	30.3	33.2	$\Sigma n_i \times d_i/N$	39
	s	3.6	4.0		
	median	29.9	32.9	d_i where $\Sigma n_i = 0.5 \times N$	
Volume distribution,	mode	30.5	33.0	d_i for max($n_i \times d_i^3$)	
	mean	31.7	34.7	$\Sigma n_i \times d_i^4 / V$	39
	s	3.9	4.4		
	median	31.2	34.1	d_i where $\Sigma n_i \times d_i^3 = 0.5 \times V$	
D_{mp}		31.2	34.2	$1/(\Sigma v_i/d_i)$	17
D_{pa}		31.8	34.8	$\bar{D}_p[(1 + 10c^2 + 15c^4)/(1 + 3c^2)]^{1/2}$	20

method used when reporting the particle sizes of chromatographic media. D_{mp} is the "mean effective diameter" used by Ladisch and Tsao[17] in the calculation of pressure drops in packed columns. The "apparent particle diameter", D_{pa}, is the estimate suggested by Nakanishi *et al.*[20] to be used in the calculation of reduced column efficiency. Thus, a conclusion that might be drawn from the data in Table II is that approximations for the particle size estimates used in the calculation of chromatographic properties may be derived from the volume distribution of the particle size of Superose 6B.

The method described here for the determination of particle size distribution is very tedious and therefore not suitable for routine quality control purposes. However, it serves to obtain accurate particle size distribution curves for the subsequent calibration of a much faster method based on the Coulter counter technique[21].

Matrix rigidity

The pressure–flow relationship for Superose 6B is shown in Fig. 2. The broken

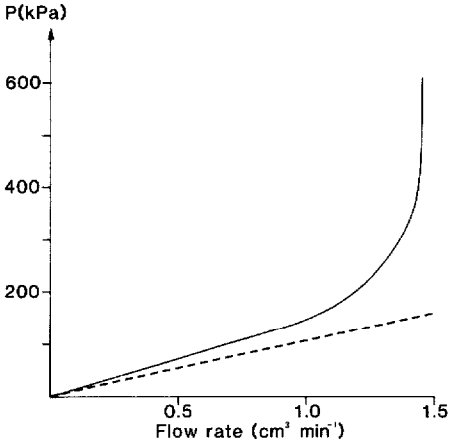

Fig. 2. Pressure–flow relationship for Superose 6B (lot 28725, $\varepsilon = 0.33$, bed dimensions 300 × 10 mm).

line represents the theoretical pressure drop as calculated from eqn. 1. The gel bed shows a linear pressure–flow behaviour at flow-rates up to 1 cm^3/min. At higher flow-rates the beads compress, the void fraction decreases and the pressure drop increases. The large pressure drop at flow-rates above 1.4 cm^3/min, *i.e.*, 3.5 MPa · min/cm^3, and the low reduction in bed volume, *i.e.*, approximately 6%, supports the theory of Ladisch and Tsao[17] that a small, highly compressed plug at the bottom of the column is responsible for the major pressure drop. Even though the beads are compressible they are not fragile and may be subjected to high pressures without deterioration in chromatographic performance. Thus, one column subjected to a pressure above 2 MPa and then allowed to decompress retained its separation properties and efficiency, *i.e.*, within ± 6%. The deviation of the pressure–flow curve in the linear region from the theoretical slope was found to be reproducible from column to column and indicated an apparent void fraction of 10% less than the one experimentally determined. A close examination of data showed that this was not caused by errors in the determination of the void fraction but is probably due to an additional pressure drop over a more densely packed region near the column outlet which was created during the packing of the column at 0.7 cm^3/min. Such a pressure drop theoretically results from a 2-cm zone of a bed with $\varepsilon = 0.20$, which would indicate a slight compression of the beads as compared to the void fraction (0.26) of close packed uniform spheres[22]. This was experimentally verified by swirling a few cm of gel at the outlet of a packed column, determining the void fraction and then switching the flow direction before running the flow gradient. This resulted in a curve with a shorter linear range but with a slope which correlated perfectly with the theoretical value. It can thus be concluded that the pressure–flow characteristics of these columns follow the Blake–Kozeny equation and there is no substantial compression of the matrix at flow-rates up to 1 cm^3/min.

Chromatographic performance

The yield of cytochrome *c* in the low ionic strength buffer was more than 95% for both batches tested. This corresponds to an apparent adsorption of less than 2 μg cytochrome *c* per millilitre bed volume.

The calibration curve for proteins is shown in Fig. 3. The working range for this gel is approximately 10^3–$4 \cdot 10^6$ Daltons. This is also illustrated by a chromatogram for the analysis of a protein mixture shown in Fig. 4. The high background is a result of the broad molecular weight distribution of Blue Dextran, *e.g.*, compare with Fig. 6. The yields of these proteins on Superose 6B are very high, see Table I. As noted on other permeation media, lysozyme is eluted at a larger elution volume than anticipated from the actual molecular weight of the protein. A rational explanation for this behaviour is provided by the "end-on insertion mechanism" where the prolate ellipsoidal protein will be eluted according to the size of its minor semiaxis if the flow-rate is sufficiently low[23].

The maximum column efficiency varied between 12,000 and 15,000 "plates per metre". The plate count depends primarily upon bead size, packing efficiency, linear flow-rate, diffusion coefficient and elution volume of the probe molecules and finally the calculation method used. The reduced plate height, *h*, where the influence of the bead size has been eliminated, is used for the evaluation of the relative efficiencies of packed columns. The inherent minimum of the reduced plate height has not yet been

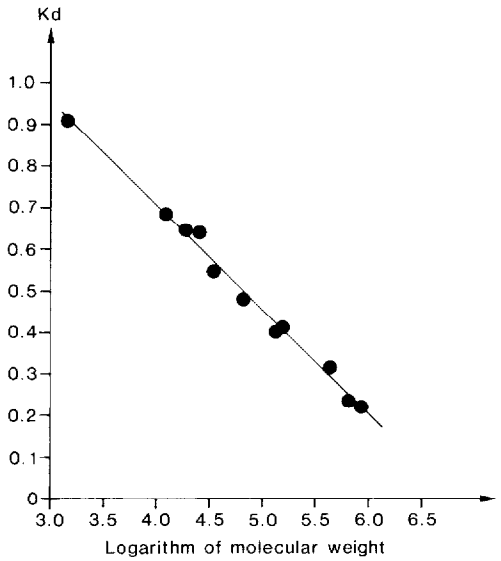

Fig. 3. Calibration curve for proteins on Superose 6B (lot 28725). The dots represent bacitracin, cytochrome *c*, myoglobin, chymotrypsinogen A, *β*-lactoglobulin, BSA, dimer of BSA, aldolase, ferritin, thyroglobulin and dimer of ferritin.

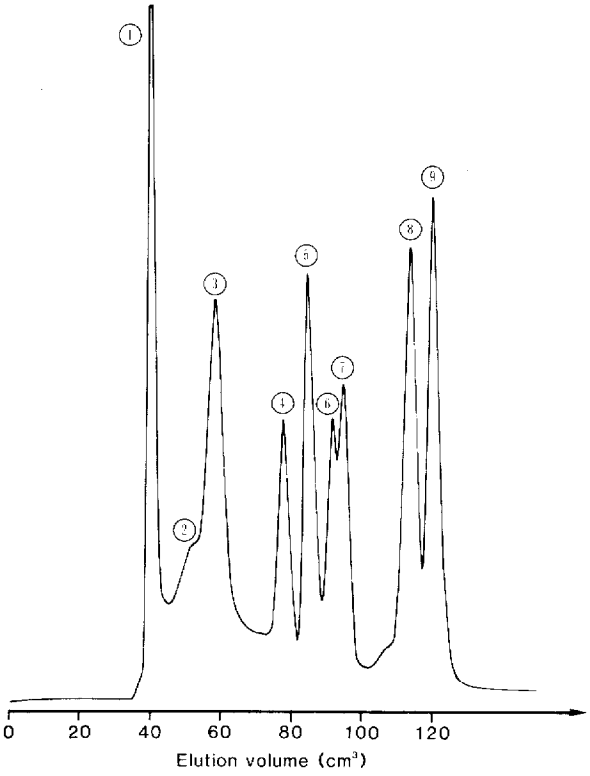

Fig. 4. Separation of a protein mixture on Superose 6B (lot 28725). Peaks: 1 = Blue Dextran 2000; 2 = dimer of thyroglobulin; 3 = monomer of thyroglobulin; 4 = BSA; 5 = *β*-lactoglobulin; 6 = myoglobin; 7 = cytochrome *c*; 8 = bacitracin and 9 = cytidine.

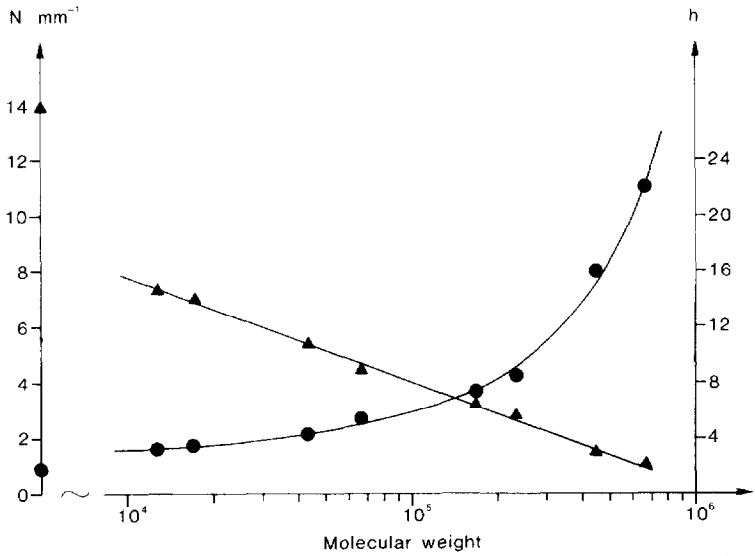

Fig. 5. Number of plates (▲) and reduced plate height (●) as a function of sample molecular weight. Solutes used are AMP, cytochrome *c*, myoglobin, ovalbumin, BSA, γ-globulin, catalase, ferritin and thyroglobulin.

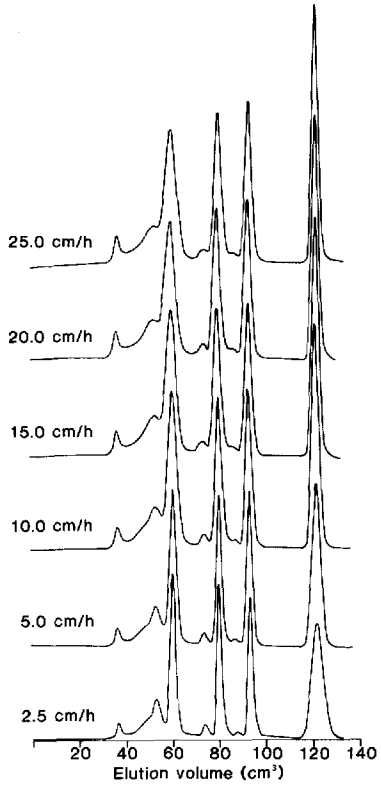

Fig. 6. Separation of thyroglobulin, BSA, myoglobin and cytidine on Superose 6B at different linear flow-rates. Bed dimensions 611 × 16 mm.

derived but it seems as though $h = 2$ is a practical attainable value[24,25]. This corresponds to 15,000 and 50,000 "plates per metre" for 33-μm and 10-μm beads respectively. However, reported efficiencies are often substantially less than the expected values[2–4]. This may be due to unfavourable test conditions, to difficulties in packing these small beads as efficiently as possible or to some special physical property of the matrices, *e.g.*, trapping of small solute molecules in long channels with dead ends.

The zone broadening of a solute is strongly dependent upon the diffusion constant and thus the solute's molecular weight[26]. The variation in column efficiency of Superose 6B for proteins of different molecular weights is shown in Fig. 5. The efficiency is drastically reduced with increased sample molecular weight and separations in the high-molecular-weight range must be performed at a low linear flow-rate to diminish the zone broadening from the slow mass transfer of these solutes. This effect is only partly reduced by the use of small beaded materials. Linear flow-rates of 2 cm/h corresponding to separation times of 48 h have been reported to be optimal on 10-μm material[14]. However, the contribution of axial dispersion to the zone broadening, especially of low-molecular-weight solutes, increases when the flow-rate decreases. These effects are illustrated in Fig. 6 where the influence of the flow-rate on the separation efficiency over the working range of Superose 6B is given. As can be

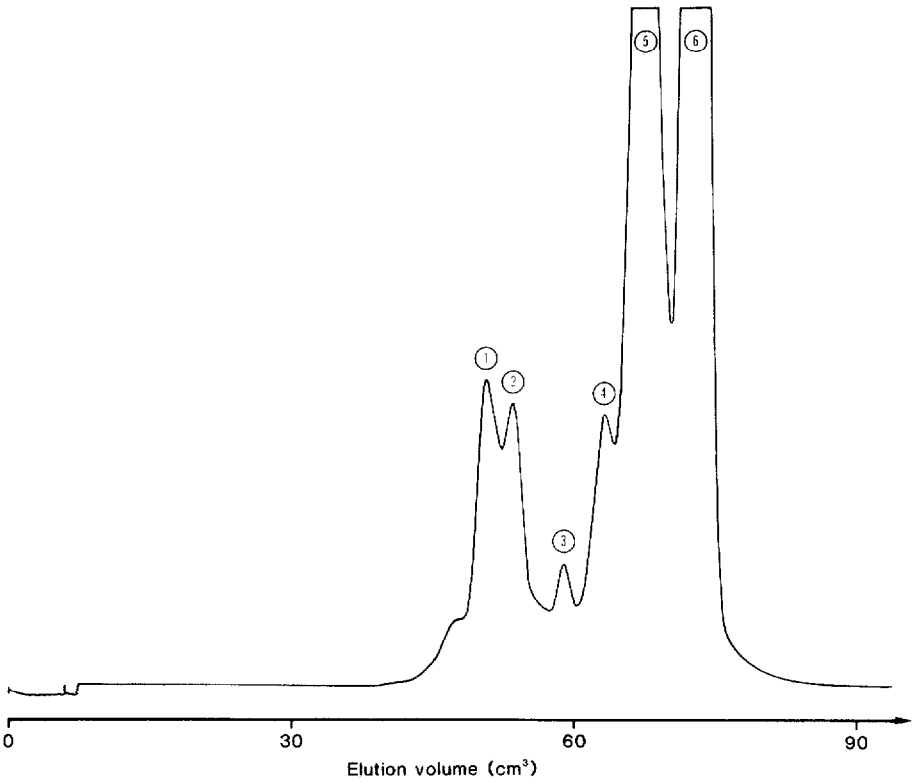

Fig. 7. Elution profile of serum on Superose 6B. Peaks: 1 = IgM; 2 = α-2-macroglobulin; 3 = not identified; 4 = IgA; 5 = IgG and 6 = albumin. Bed dimensions 546 × 16 mm. Linear flow-rate 1.5 cm/h.

expected, the largest effects are noted for the outer part of the separation range while the separation efficiency at the middle of the working range, *e.g.*, 20,000–70,000 Daltons, is comparatively high even at the highest flow-rate. In essence, Fig. 6 shows that the general principle of liquid chromatography, *i.e.*, there is more separation efficiency to gain from increasing the selectivity than from increasing the plate number, is also applicable to permeation chromatography.

Whereas the plate count is related to the broadening of the sample zones, the separation of the peaks is more critically affected by the porosity and the permeability (ratio of pore volume, V_i, to interstitial volume, V_0) of the material. The porosity is selected to yield an appropriate working range of the gel, but the permeability is an inherent property of the matrix. The permeability of Superose 6B is approximately 2.3 as compared to 0.6–1.4 for many other commercial high-performance size exclusion chromatographic columns[3]. Theoretically, this means that the latter columns must yield more than twice as many plates to compensate for the lower permeabilities, if corresponding resolution is to be achieved[27]. This was experimentally verified in a study of μBondagel (V_i/V_0 = 0.84, N = 6000) and Sepharose CL 6B (V_i/V_0 = 2.2, N = 2000)[28] and may also be illustrated by the comparison of the separation in Fig. 4 with that of a similar mixture on a 10-μm material[29].

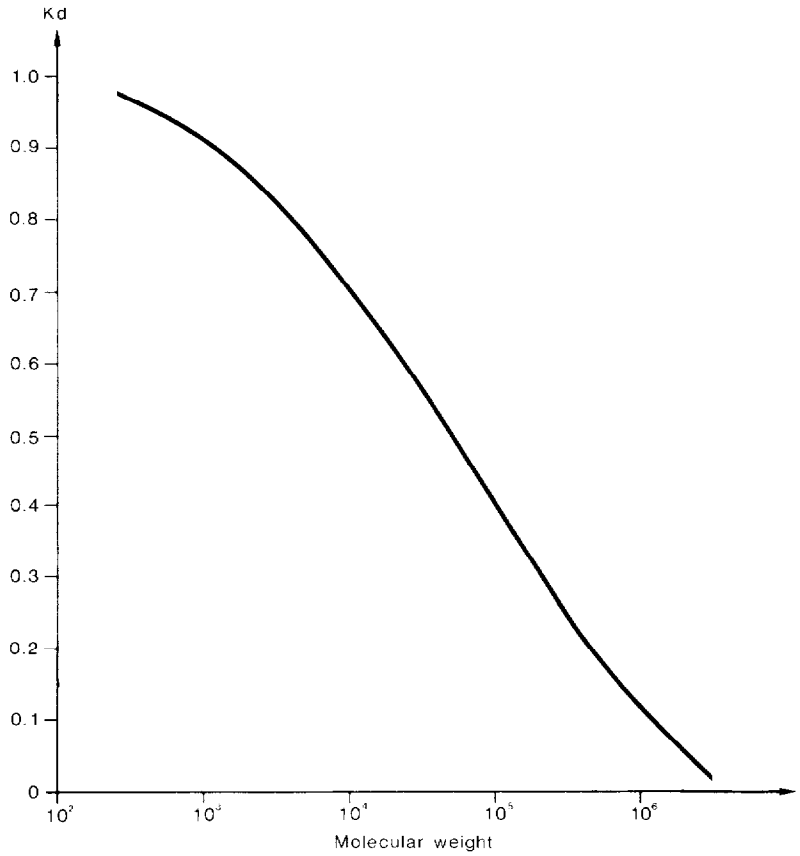

Fig. 8. Calibration curve for dextrans on Superose 6B.

The high permeability of the matrix enables high sample loads (more than 0.2 g) and makes this gel very suitable for high resolution preparative gel filtration[2,30]. The separation power of Superose 6B is illustrated in Fig. 7 which shows the elution profile of a pooled serum sample. The peaks were collected and their identity was confirmed by immunodiffusion[31].

Analytical gel filtration, as outlined in the Nordic Pharmacopoeia using Sepharose 4B[32,33], has gained international acceptance as a fast and reliable method for the determination of molecular weight distribution (MWD) curves of clinical dextrans[34–37]. As can be seen from the calibration curve for dextrans on Superose 6B, in Fig. 8, this gel is suitable for the MWD assay of dextrans from 10^3 to 10^6 Daltons. This curve was obtained from the elution profiles of T-dextrans shown in Fig. 9 with a previously described procedure[32]. Furthermore, by using this smaller beaded material the time of analysis could be reduced from 20[33] or 6 h[13] to 2 h[18]. This method has been proposed as a reference method for the assay of MWDs of clinical dextrans[38].

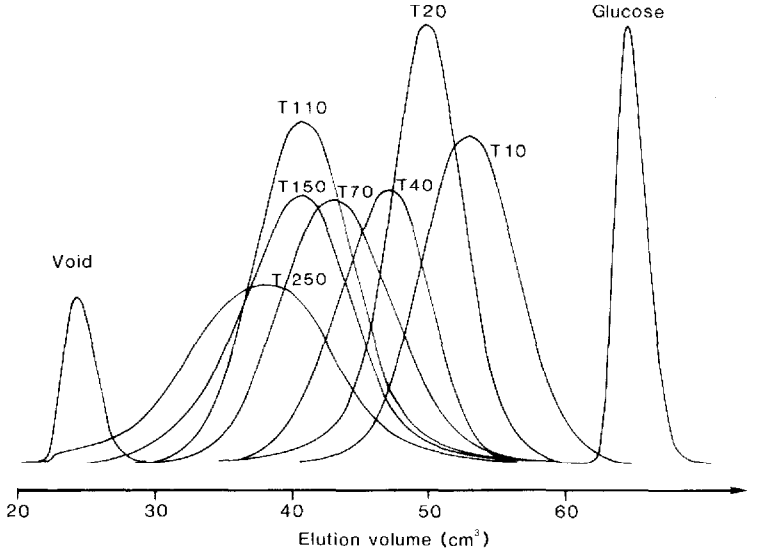

Fig. 9. Elution profiles of dextran standards on Superose 6B.

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REFERENCES

1 H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 409.
2 T. W. Hearn, F. E. Regnier and C. T. Wehr, *Amer. Lab.*, October (1982) 18.
3 E. Pfannkoch, K. C. Lu, F. E. Regnier and H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 430.
4 S. Rokushika, T. Ohkawa and H. Hatano, *J. Chromatogr.*, 176 (1979) 456.
5 B. Renck and R. Einarsson, *J. Chromatogr.*, 197 (1980) 278.

6 R. A. Jenik and J. W. Porter, *Anal. Biochem.*, 111 (1981) 184.
7 M. Gurkin and V. Patel, *Amer. Lab.*, January (1982) 64.
8 F. Hefti, *Anal. Biochem.*, 121 (1982) 378.
9 S. Aoyagi, K. Hirayanagi, T. Yoshimura and T. Ishikawa, *J. Chromatogr.*, 253 (1982) 133.
10 W. Kopaciewicz and F. E. Regnier, *Anal. Biochem.*, 126 (1982) 8.
11 P. L. Dubin and I. J. Levy, *J. Chromatogr.*, 235 (1982) 377.
12 Y. Kato and T. Hashimoto, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 45.
13 L. Hagel, *J. Chromatogr.*, 160 (1978) 59.
14 J. Sjödahl, *Lab. Pract.*, July (1981) 717.
15 S. Hjertén and Y. Kunquan, *J. Chromatogr.*, 215 (1981) 317.
16 L. Hagel and A. Bergvall, Pharmacia Fine Chemicals, Uppsala, unpublished results.
17 M. R. Ladish and G. T. Tsao, *J. Chromatogr.*, 166 (1978) 85.
18 K. Granath, Department of Research, Pharmacia Pharmaceuticals, Uppsala, personal communication.
19 L. Hagel, *Anal. Chem.*, 50 (1978) 569.
20 K. Nakanishi, S. Yamamoto, R. Matsumo and T. Kamikubo, *Agr. Biol. Chem.*, 42 (1978) 1943.
21 R. Andersson, in M. J. Groves (Editor), *Particle Size Analysis*, Heyden, London, 1978, p. 411.
22 P. G. Squire, A. Magnus and M. E. Himmel, *J. Chromatogr.*, 242 (1982) 255.
23 S. C. Meredith and G. R. Nathans, *Anal. Biochem.*, 121 (1982) 234.
24 P. A. Bristow and J. H. Knox, *Chromatographia*, 10 (1977) 279.
25 R. W. Stout, J. J. DeStefano and L. R. Snyder, *J. Chromatogr.*, 261 (1983) 189.
26 J. C. Giddings and K. L. Mallik, *Anal. Chem.*, 38 (1966) 997.
27 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 125 (1976) 103.
28 L. Hagel, *Abstracts of Uppsala Dissertations from the Faculty of Science, 453*, Almqvist & Wiksell, Stockholm, 1978.
29 Y. Kato, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 190 (1980) 297.
30 L. Hagel and T. Andersson, presented at *Third Int. Symp. on HPLC of Proteins, Peptides and Polynucleotides, Monte Carlo, November 1983.*
31 G. Mancini, A. O. Carbonara and J. F. Heremans, *Immunochemistry*, 2 (1965) 235.
32 K. A. Granath and B. E. Kvist, *J. Chromatogr.*, 28 (1967) 69.
33 G. Nilsson and K. Nilsson, *J. Chromatogr.*, 101 (1974) 137.
34 A. M. Basedow and K. H. Ebert, *Infusionstherapie*, 2 (1975) 261.
35 H. Pitz, *Anaesthetist*, 24 (1975) 500.
36 R. M. Alsop, G. A. Byrne, J. N. Done, I. E. Earl and R. Gibbs, *Process Biochem.*, December (1977) 15.
37 P. E. Barker, B. W. Hatt and G. J. Vlachogiannis, *J. Chromatogr.*, 208 (1981) 74.
38 K. Nilsson, Pharmacia Infusion AB, personal communication.
39 T. Allen, *Particle Size Measurement*, Chapman & Hall, London, 1981, p. 128.