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Chemical, physical and chromatographic properties of Superdex 75 prep grade and Superdex 200 prep grade gel filtration media

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ABSTRACT

Two novel gel filtration media, Superdex 75 prep grade and Superdex 200 prep grade are described. The new media have high selectivities similar to those of Sephadex G-75 and G-200, respectively, but permit the use of high flow-rates as with cross-linked agarose beads. They consist of cross-linked agarose beads modified with dextran to obtain the desired selectivity. The mean particle size is *ca.* 33 μm (30–36 μm) and a reduced plate height of *ca.* 2.0 is regularly obtained in packed columns. A flow-rate of 50 cm/h can be used even in large columns. The media are chemically stable at pH 3–14 and can be used with a wide range of eluents. The performance of the media with respect to flow-rate, sample load and sample concentration for given model systems is described. Superdex 75 prep grade was used to separate monomeric forms of a recombinant fusion protein (ZZ–brain insulin-like growth factor molecular weight 22 000 dalton) from dimers and oligomers. Superdex 200 prep grade was used to obtain baseline separation of a monoclonal antibody from antibody dimer and contaminating transferrin.

INTRODUCTION

In 1959, Sephadex gels were introduced as the first media for the gel filtration of water-soluble macromolecules [1,2]. Since then, a host of chromatographic supports for gel filtration have become available from a number of sources. The technique has been reviewed several times, *e.g.*, see papers by Hagel [3] and Fischer [4] and references cited therein.

Sephadex represents a class of gels termed xerogels. These are characterized by complete collapse of the gel structure on removal of solvent (water) [5–7]. In fact, such gels can, in the swollen state, be regarded as elastic solutions as opposed to ordinary viscous solutions. Cross-linked polyacrylamide gels such as those of the Bio-Gel P series belong to the same type of gels. Chromatographically they are characterized by high selectivities, *i.e.*, large differences in partition coefficients (K_D) for proteins or other macromolecules of different molecular weight.

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The main drawback with these kinds of gels is their limited rigidity, which causes the gel particle to deform when exposed to drag forces generated by liquid flow in a chromatographic column. Gels with permanent pores (aerogels), such as porous glass, are not deformed and allow much higher flow-rates to be used. Many so-called macroreticular gels, used for the chromatography of biological macromolecules, are xerogel–aerogel hybrids. Gels based on agarose belong to this category, in addition to many gels based on synthetic polymers. The aerogel element in these gels confers high flow resistance but they suffer from comparatively low selectivity. It seems that the limitation with regard to selectivity is inherent in the macroreticular structure of these materials.

Superdex 75 prep grade and Superdex 200 prep grade combine the high selectivity of the xerogels with the high flow resistance of the xerogel–aerogel hybrids. The Superdex gels are made by a proprietary method in which cross-linked agarose beads provide the required mechanical strength. Dextran is incorporated into the macropores of the beads by covalent binding of the polysaccharide chains to the surface of the walls of the pores and to the periphery of the beads. The immobilized chains of dextran behave in water as an immobilized polysaccharide solution and afford a gel filtration phase similar to that in beads of Sephadex.

The use of composite materials similar to the Superdex gels is not new. Ultrogel AcA supports (Reactifs IBF, France) are claimed to receive their mechanical strength from an agarose matrix encompassing an acrylamide polymer which provides high resolution in gel filtration. Sephacryl beads (Pharmacia LKB Biotechnology, Sweden) are also composite materials. They are made by cross-linking allyldextran with N,N-methylenebisacrylamide and a core structure of polymerized N,N-methylene-bisacrylamide formed in this process is assumed to be the major contributor to the rigidity of the particles.

In 1987, Hjertén *et al.* [8] reported a downward shift of the exclusion limit of agarose gels when coupling dextran to residual vinyl groups after cross-linking of the gel with divinyl sulphone.

A separate paper [9] described the performance of Superdex 75 prep grade and Superdex 200 prep grade in buffers of different pH and ionic strengths and after repeated treatment with 1 *M* sodium hydroxide or 0.1 *M* hydrochloric acid. Superdex 200 prep grade and Superdex 75 prep grade have been used in the purification of a fusion protein containing a malaria antigen [10], in the large-scale purification of staphylococcal enterotoxin B [11] and in the development of a production system for recombinant human superoxide dismutase [12].

EXPERIMENTAL

Nomenclature and calculations

The following nomenclature and equations were used to characterize columns and chromatographic resolution:

V_0	Void volume of column
V_c	Geometric volume of column
V_p	Pore volume of column
V_s	Support (matrix) volume of column
V_t	Total liquid volume of column

V_p/V_0	Permeability
V_R	Retention (elution) volume
$V_{R,n}$	Retention volume of peak No. n
K_D	Distribution coefficient for a solute passing through a gel filtration column
K_{av}	Fraction of stationary gel volume accessible to a given solute
N	Number of theoretical plates
R_s	Chromatographic resolution
$W_{b,n}$	Width of peak No. n at base of peak
MW	Molecular weight
W_h	Peak width at half-height

$$V_c = V_0 + V_s + V_p$$

$$V_{s,rel} = \frac{V_s}{V_p + V_s}$$

$$V_{p,rel} = \frac{V_p}{V_p + V_s}$$

$$K_D = \frac{V_R - V_0}{V_t - V_0}$$

$$K_{av} = \frac{V_R - V_0}{V_c - V_0}$$

$$N = 5.54(V_R/W_h)^2$$

$$R_s = \frac{2(V_{R,n+1} - V_{R,n})}{W_{b,n+1} + W_{b,n}}$$

Chemicals

Superdex 75 prep grade, Superdex 200 prep grade, Sephadex G-75 and Sephadex G-200 were supplied by Pharmacia LKB Biotechnology. A partly purified sample of ZZ–brain IGF (recombinant brain insulin-like growth factor fused to an IgG-binding peptide) was kindly provided by KabiGen (Stockholm, Sweden) as a freeze-dried powder. Substances used in the evaluation of the chromatographic performance of the supports are listed in Table I.

Equipment

All equipment was obtained from Pharmacia LKB Biotechnology unless specified otherwise.

(i) Determination of selectivity curves: FPLC system, HR 10/30 columns, RefractoMonitor III (Laboratory Data Control, Riviera Beach, FL, U.S.A.), Optilab 901 (Tecator, Höganäs, Sweden). (ii) Gel filtration of protein mixtures (Fig. 3): P-3500 pump, BioPilot column, Rec. 481 recorder, UV-M detector with 2-mm cell.

TABLE I

SUBSTANCES USED FOR EVALUATION OF CHROMATOGRAPHIC PERFORMANCE

Substance	Molecular weight	Isoelectric point	Source
Blue Dextran 2000	2 000 000		Pharmacia
Thyroglobulin	669 000		Pharmacia
Ferritin	440 000		Pharmacia/Sigma
Aldolase	158 000		Pharmacia
Immunoglobulin G (IgG)	156 000		Pharmacia/Sigma
Transferrin	77 000		Kabi/Sigma
Bovine serum albumin (BSA)	67 000	*ca.* 5	Pharmacia/Sigma
Ovalbumin	43 000	4.7	Pharmacia
Chymotrypsinogen A (Chym A)	25 000		Pharmacia
Myoglobin	18 800		Sigma
Ribonuclease (RNase)	13 700	8.9	Pharmacia
Cytochrome *c* (Cyt *c*)	12 600	*ca.* 9.2	Sigma
Aprotinin	6500		Fluka/Sigma
Vitamin B_{12}	1400		Sigma
Raffinose	594		
Cytidine	243		Fluka
Glucose	180		
Deuterium oxide	20		Merck
Dextran	[a]		Pharmacia

[a] Mean molecular weight of dextran fractions (Pharmacia): 276 500, 196 300, 123 600, 66 700, 43 500, 21 400, 9890, 4440, 1080 and 594.

(iii) Study of chemical stability (Fig. 5): P-500 pump, V-7 injector with 200-μl loop, UV-1 detector with HR 10-mm cell, Rec. 481 recorder. (iv) Study of the influence of flow-rate, sample size and protein load: BioPilot system including a UV-M detector in work with Superdex 75 prep grade and FPLC system with a UV-1 or UV-2 detector in work with Superdex 200 prep grade. (v) Purification of ZZ–brain IGF: P-500 pump, MV-7 valve, UV-M detector, REC-482 recorder, FRAC-100 fraction collector, LCC-500 control unit, SpeedVac freeze-drier (Savant) and PhastSystem for electrophoresis. (vi) Purification of IgG_{2a} monoclonal antibody: BioPilot system and PhastSystem. (vii) Particle size analysis: Coulter Counter ZM equipped with C-256 pulse-height analyser and calibrated with Superdex samples measured by microscope analysis [13,14].

Chromatographic and electrophoretic methods

Details regarding chromatographic conditions are given in the figure legends. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed under non-reducing conditions on PhastGel Gradient 8–25. The separations and the Commassie brilliant blue and silver staining procedures were carried out according to the PhastSystem manual. Other conditions are given in the figure legends.

RESULTS

Selectivity, fractionation range and other physical and chemical properties

We used deuterium oxide as a marker for the total liquid volume of columns (V_t). This has been shown to give an overestimate of the V_t value owing to isotope exchange with hydroxyl hydrogens of the gels [15]. No corrections were made for this effect.

In Fig. 1, K_D values of proteins and vitamin B_{12} on Superdex 75 prep grade and Superdex 200 prep grade are presented together with corresponding data for Sephadex G-75 and Sephadex G-200. The amount of dextran incorporated in the beads of Superdex was adjusted to match the selectivities of Sephadex. It is evident that both the steepness of the selectivity curves and the exclusion limits are in good agreement when corresponding Superdex and Sephadex media are compared.

Selectivities as measured by K_{av} for dextran fractions complemented with raffinose and glucose on Superdex 75 prep grade and Superdex 200 prep grade are shown in Fig. 2.

Chromatography of mixtures of test proteins is shown in Fig. 3. Over a major part of the separation ranges, proteins with a difference in molecular weight of less than a factor of 2 could be separated. With Superdex 75 prep grade a factor of 1.5 will be sufficient in many instances.

Table II lists some properties of Superdex. In order to achieve a high performance in gel filtration, it is essential to keep the pore volume as high as possible,

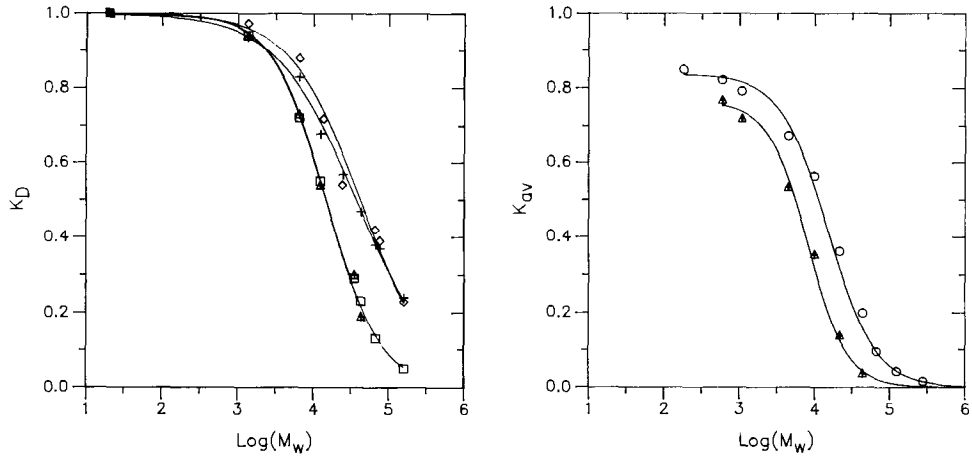


Fig. 1. K_D of globular proteins and vitamin B_{12} *versus* log MW [log (M_w)] on (□) Superdex 75 prep grade, (+) Superdex 200 prep grade, (△) Sephadex G-75 and (◇) Sephadex G-200. Experiments with deuterium oxide as sample and distilled water as eluent were carried out to determine V_t. Buffer for chromatography of proteins and vitamin B_{12}: 50 m*M* sodium phosphate–100 m*M* sodium chloride (pH 7.4). Column: HR 10/30. Flow-rates: 0.50 ml/min for Superdex gels, 0.10 or 0.25 ml/min for Sephadex gels. Detection: absorbance at 280 nm for proteins and refractive index (RefractoMonitor III) for deuterium oxide. Sample: deuterium oxide, 200 μl; solution of proteins, vitamin B_{12} and cytidine in elution buffer, 200 μl.

Fig. 2. K_{av} of dextran fractions, raffinose and glucose on (△) Superdex 75 prep grade and (○) Superdex 200 prep grade *versus* log MW. Columns: 600 × 26 mm I.D. Eluent: 250 m*M* sodium chloride and 0.02% sodium azide in water. Flow-rate: 5 ml/min. Detection: Optilab 901.

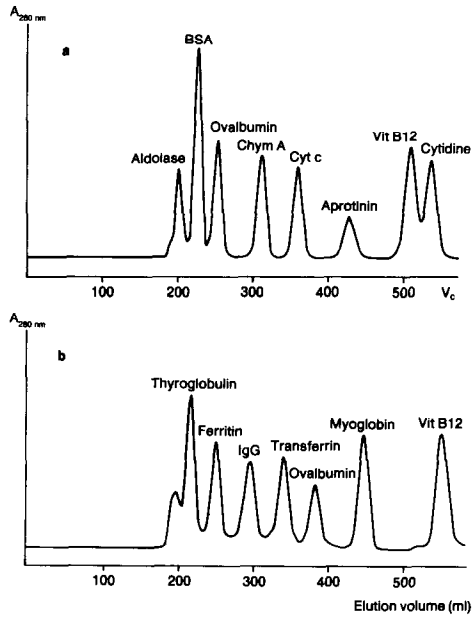


Fig. 3. Chromatography of test mixtures on (a) Superdex 75 prep grade and (b) Superdex 200 prep grade. Column: BioPilot 35/600. Flow-rate: (a) 15 cm/h (2.4 ml/min); (b) 10 cm/h (1.6 ml/min). Buffer: 50 m*M* sodium phosphate–150 m*M* sodium chloride–0.01% sodium azide (pH 7.0). Sample size: 1.0 ml. Total concentration of test substances: (a) 23.7 mg/ml; (b) 21.1 mg/ml. V_0 was determined in a separate run with Blue Dextran 2000, 2 mg/ml in buffer, sample size 1.0 ml.

i.e., the matrix volume should be kept to a minimum. It has been possible to reach the exclusion limits with much lower support volumes than would be possible by making conventional macroporous gels.

Particle size distributions are given in Fig. 4. A reduced plate height of *ca.* 2.0 in packed columns has been routinely achieved.

For wide-bore columns (wider than 30 mm), prepacked columns are guaranteed for use at flow-rates up to 50 cm/h. In narrower columns, flow-rates of up to 300 cm/h

TABLE II

SOME PROPERTIES OF SUPERDEX; TYPICAL VALUES

Property	Superdex 75 prep grade	Superdex 200 prep grade
Particle size $d_{50,v}$	33 μm	33 μm
Relative matrix volume ($V_{s,rel}$)	11%	6%
Void volume fraction (V_0/V_c)[a]	~30%	~30%
Permeability (V_p/V_0)[a]	2.1	2.2
N (plates/m)	15 000–20 000	15 000–20 000
Approximate separation range, proteins (dalton)	3000–70 000	3000–600 000

[a] In BioPilot 35/600 and 60/600 columns.

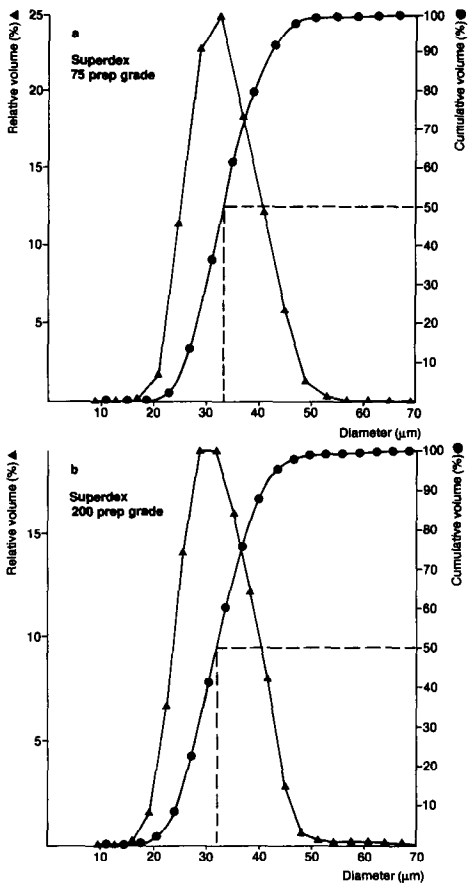


Fig. 4. Particle size distribution of typical lots of Superdex 75 prep grade and Superdex 200 prep grade.

have been used without any marked compression of the gel beds (data not shown). The influence of flow-rate on gel filtration results is discussed below.

The stability of the gels on storage in various aqueous solutions at 40°C for 1 week was studied by measuring K_{av} before and after storage (Fig. 5). An increase in the K_{av} value would indicate that some of the dextran has been removed from the pores. In most instances no change in K_{av} was observed. In 0.5 *M* sodium hydroxide solution a slight increase was noted. However, as shown in a more detailed study, repeated cleaning in place and sanitization of columns with sodium hydroxide solution had no significant influence on performance [9]. Autoclaving once did not cause any noticeable change in K_{av}, but after five autoclaving cycles a minor increase was observed.

Influence of flow-rate, sample load and sample volume on chromatographic performance

The chromatographic performance of Superdex 75 prep grade was studied using solutions of cytochrome *c* (Cyt *c*), chymotrypsinogen A (Chym A) and bovine serum

albumin (BSA). Resolution was calculated for the Cyt *c*–Chym A pair in experiments where the proteins were run separately on the columns. The results are shown in Figs. 6 and 7.

A more elaborate study was carried out with Superdex 200 prep grade (Figs. 8–15). In this instance a mixture of IgG and transferrin was used. Peaks eluting before the IgG peak represent impurities.

The results show that under conditions normally used in preparative work, flow-rate has only a minor effect on resolution, which corroborates results published by other workers [16] (Figs. 6, 8 and 9). Only with the higher molecular weight proteins used in the experiments with Superdex 200 prep grade was a substantial increase in resolution observed on going to very low flow-rates. The results suggest that it is possible to use the specified maximum flow-rate of 50 cm/h in many preparative applications of the gels.

The influence on resolution of sample load as determined by sample concentration and sample volume has been discussed by several workers [4,17,18]. We found that the use of high sample concentrations to obtain high sample loads (up to *ca.* 1 g of protein per litre of gel) had only a small negative effect on resolution (Figs. 10 and 11).

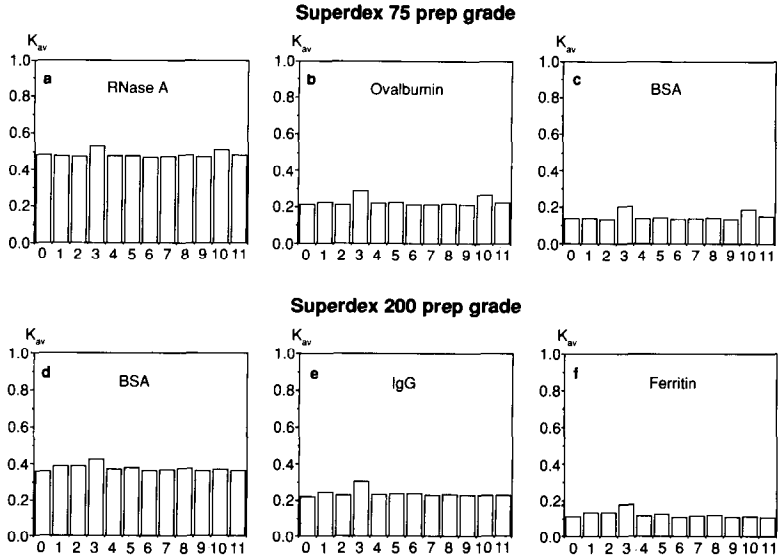


Fig. 5. Stability of Superdex 75 prep grade and Superdex 200 prep grade measured as change in K_{av} of proteins on storage in various media and after autoclaving. Storage media or autoclaving cycles: 0, control; 1, 1.0 *M* acetic acid; 2, 50 m*M* sodium phosphate, 2.0 *M* NaCl (pH 7.0); 3, 0.5 *M* NaOH; 4, 30% isopropanol; 5, 30% acetonitrile; 6, 2% SDS; 7, 6.0 *M* guanidinium · HCl; 8, 8.0 *M* urea; 9, 24% ethanol; 10, autoclaving five times; 11, autoclaving once. Storage test (1–9): the gel was washed with the storage medium on a glass filter and kept in the medium at 40°C for 7 days (experiments 1–8) and 4 weeks (experiment 9). Autoclaving: experiments 10 and 11, 121°C for 20 min. After storage or autoclaving the gels were washed with distilled water and packed in HR 16/50 columns for chromatographic testing. Chromatographic conditions: 200 µl of a mixture of three proteins were applied and eluted at a flow-rate of 15 cm/h (Superdex 75 prep grade) or 10 cm/h (Superdex 200 prep grade). Buffers as in Fig. 2. V_0 was determined in a separate run with Blue Dextran 2000, 2 mg/ml in buffer, sample size 1.0 ml.

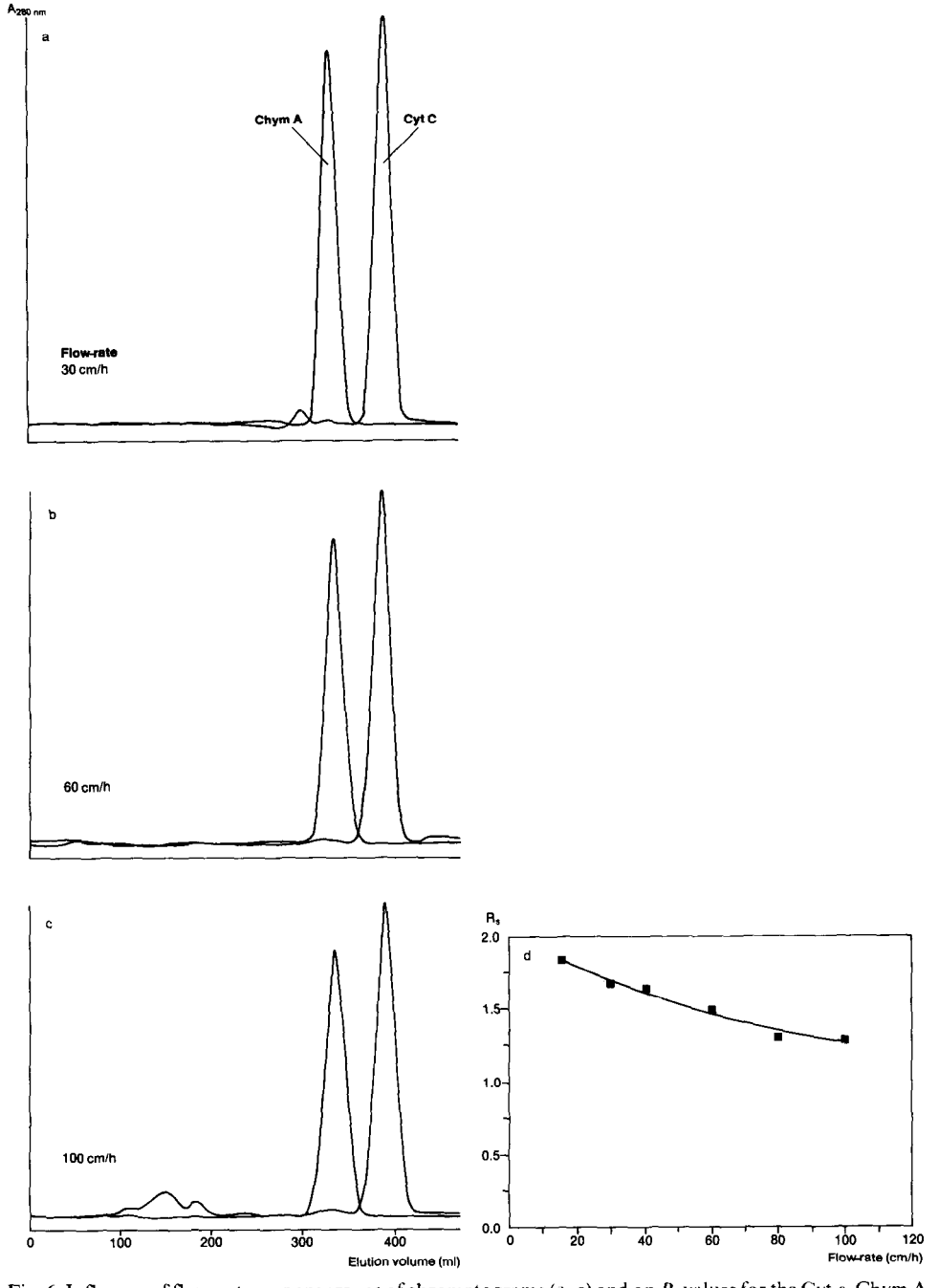


Fig. 6. Influence of flow-rate on appearance of chromatograms (a–c) and on R_s values for the Cyt *c*–Chym A pair (d) at constant sample load and sample volume with Superdex 75 prep grade. Column: 600 × 35 mm I.D., N = 15 700 plates/m (acetone). Buffer: 50 m*M* sodium phosphate–100 m*M* NaCl (pH 7.2). Sample concentration: Cyt *c* 2 mg/ml, Chym A 2 mg/ml. Sample volume: 16.5 ml (3% of V_c). Each protein was run separately.

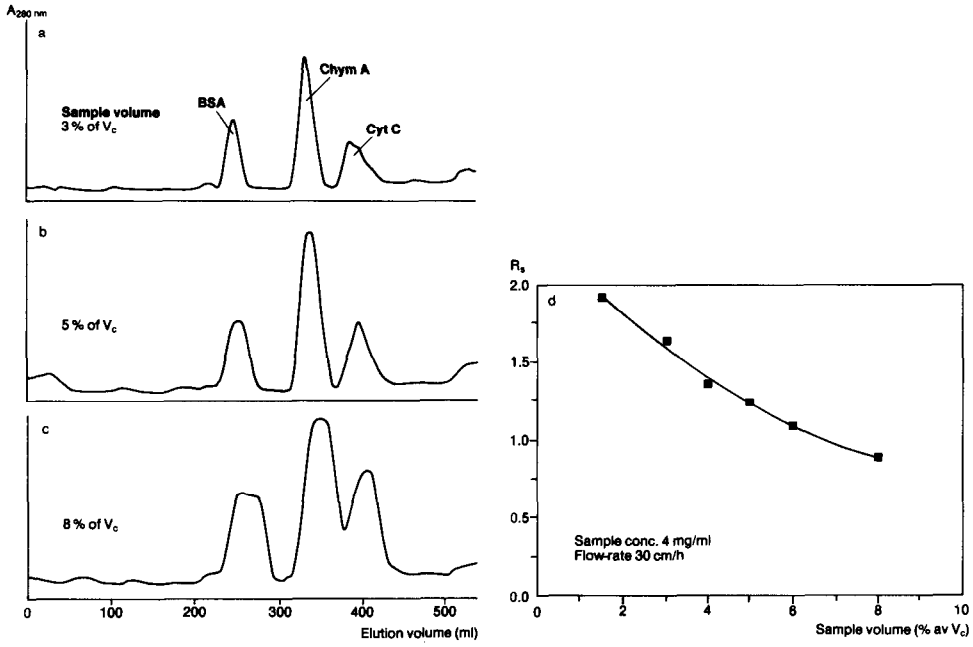


Fig. 7. Influence of sample volume on appearance of chromatograms (a–c) and on R_s values for the Cyt *c*–Chym A pair (d) at constant sample concentration and constant flow-rate with Superdex 75 prep grade. Flow-rate: 4.8 ml/min (30 cm/h). In a–c, a mixture of Cyt *c* (2 mg/ml), Chym A (2 mg/ml) and BSA (6 mg/ml) was used as a sample. In d, Cyt *c* and Chym A (both 2 mg/ml) were chromatographed separately. Other conditions as in Fig. 6.

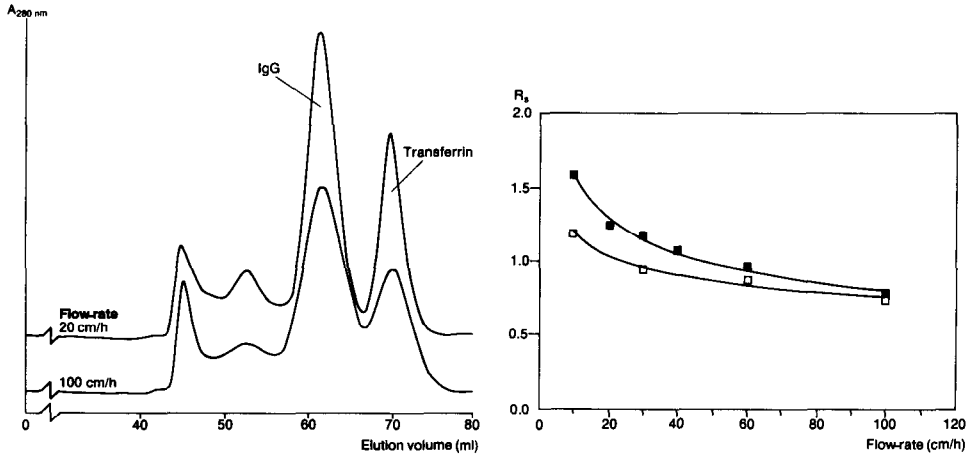


Fig. 8. Chromatography on Superdex 200 prep grade at high and low flow-rates with constant volume (0.8% of V_c) and constant concentration of sample (8 mg protein/ml). Column: 600 × 16 mm I.D., N = 18 500 plates/m. Buffer: 50 m*M* sodium phosphate–100 m*M* NaCl (pH 7.2). Sample: equal amounts of transferrin and IgG.

Fig. 9. Influence of flow-rate on resolution on Superdex 200 prep grade with constant sample volume (0.8% of V_c) and at two levels of sample load (■ = 70; □ = 530 mg/l gel). Concentration of sample: 8 and 64 mg protein/ml. Other conditions as in Fig. 8.

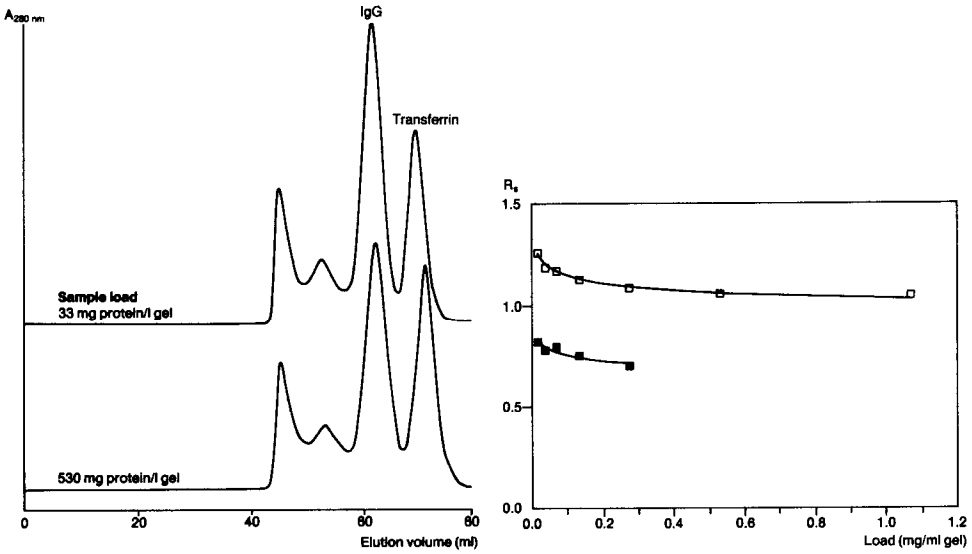


Fig. 10. Chromatography on Superdex 200 prep grade at high and low sample concentrations. Sample volume: 0.8% of V_c. Flow-rate: 30 cm/h. Other conditions as in Fig. 8.

Fig. 11. Influence of sample load on resolution on Superdex 200 prep grade at two levels of sample volume (□ = 0.8%; ■ = 3.3% of V_c). Flow-rate: 30 cm/h. Other conditions as in Fig. 8.

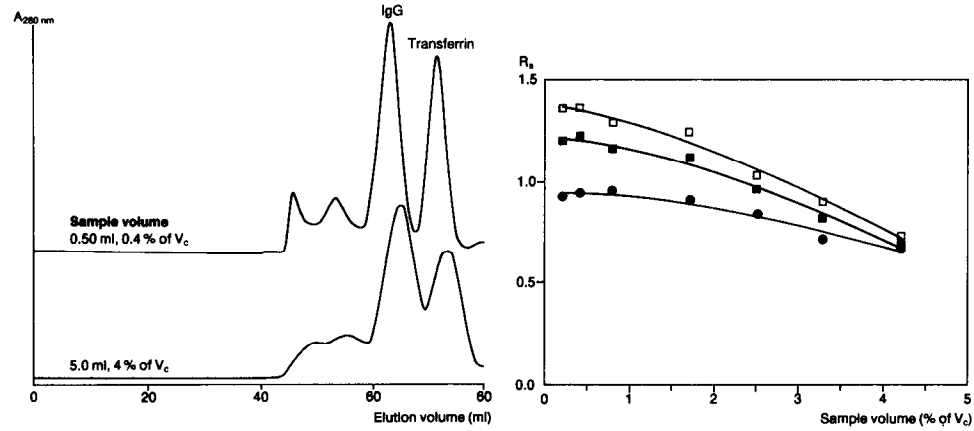
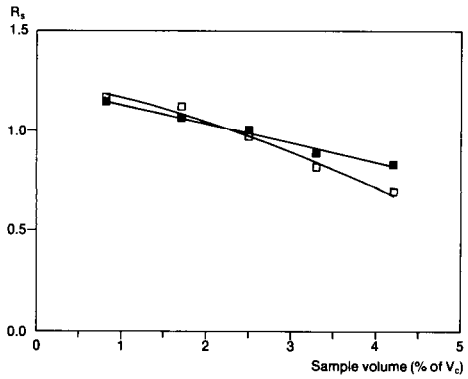


Fig. 12. Chromatography on Superdex 200 prep grade at high and low sample volumes. Sample load: 70 mg/l gel. Flow-rate: 30 cm/h. Other conditions as in Fig. 8.

Fig. 13. Influence of sample volume on resolution on Superdex 200 prep grade at three different flow-rates (□ = 15; ■ = 30; ● = 60 cm/h) and at constant sample (protein) load (70 mg/l gel). Other conditions as in Fig. 8.



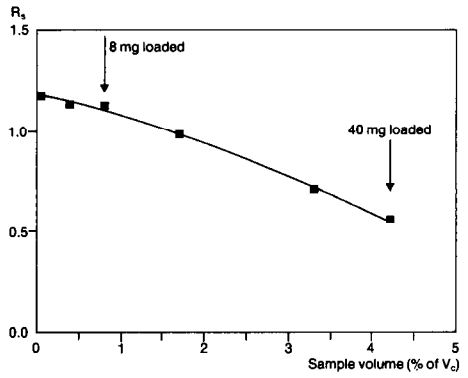


Fig. 14. Influence of sample volume on resolution on Superdex 200 prep grade at two levels of constant protein load (□ = 70; ■ = 530 mg/l gel) and at constant flow-rate. Other conditions as in Fig. 8.

Fig. 15. Influence of sample volume on resolution on Superdex 200 prep grade at constant flow-rate and constant sample concentration. Flow-rate: 30 cm/h. Sample concentration: 8 mg protein/ml. Other conditions as in Fig. 8.

Of the parameters studied, sample volume had the greatest effect on resolution (Figs. 7 and 12–15). The effect was more pronounced when low flow-rates were used (Fig. 13). Fig. 14 shows that R_s was approximately the same at two levels of protein loads, 70 and 530 mg/l gel, when the sample volume was varied. Indications are that at high sample volumes the higher protein load gives higher R_s values. Fig. 15 shows the results of experiments in which the amount of protein increased in proportion to the sample volume. The progression of the resolution *versus* sample volume curve is similar to that obtained at constant protein load and at the same flow-rate in Fig. 13.

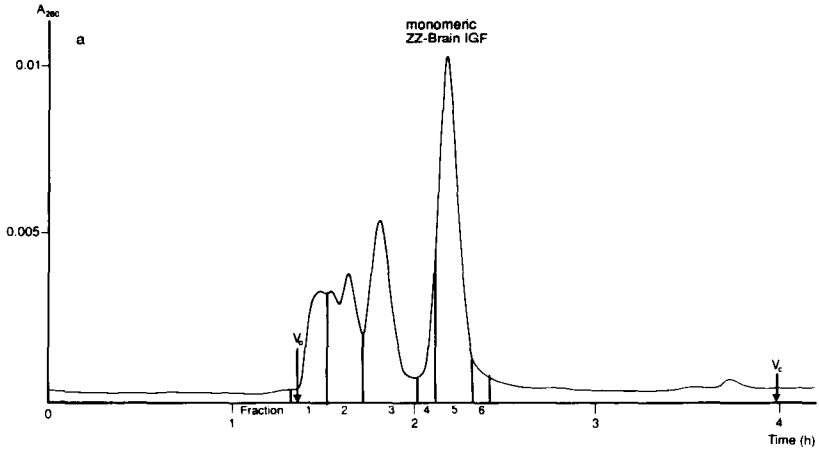
Purification of ZZ–brain IGF on Superdex 75 prep grade

Brain insulin-like growth factor (brain IGF) is a truncated form of IGF-1, lacking the N-terminal tripeptide, and is considered to be important for the growth and metabolism of the central nervous system. It has been expressed as a recombinant extracellular fusion protein at KabiGen. The fusion partner [19], designated ZZ, is a synthetic IgG-binding protein derived from staphylococcal protein A. The complete recombinant fusion protein is called ZZ–brain IGF. The initial purification steps, not shown here, were affinity chromatography on IgG Sepharose Fast Flow and ion-exchange chromatography.

The purpose of the gel filtration step was to remove dimers and oligomers from the monomeric ZZ–brain IGF, MW 22 000 dalton. As seen in Fig. 16, dimers and larger aggregates could be efficiently eliminated by gel filtration on Superdex 75 prep grade and a significant degree of separation of dimers and trimers was also obtained.

Purification of monoclonal antibodies on Superdex 200 prep grade

Fig. 17 shows the final purification of a monoclonal antibody from hybridoma cell culture supernatants after concentration by ultrafiltration and initial purification by cation-exchange chromatography. The principal contaminants were antibody dimer (peak 1) and transferrin (peak 3). The purity of the monoclonal antibody was >98% after the gel filtration step as determined by electrophoresis.



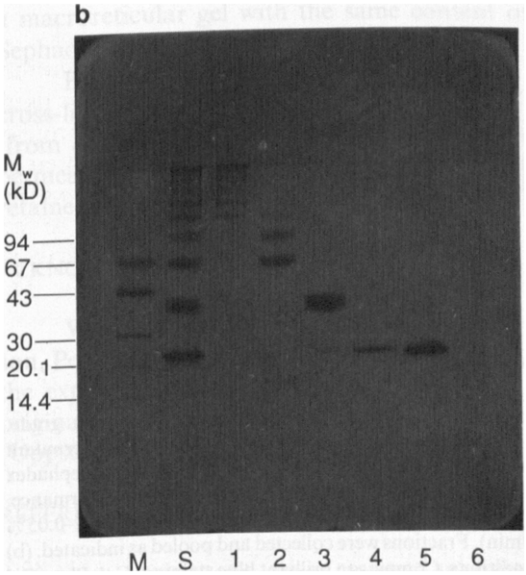


Fig. 16. Gel filtration of partly purified ZZ–brain IGF on Superdex 75 prep grade to separate the monomeric form, MW 22 000 dalton, from dimers and oligomers. (a) Chromatography. Column: 600 × 16 mm I.D. Buffer: 0.3 *M* ammonium acetate (pH 6.0). Flow-rate: 15 cm/h (0.5 ml/min). Sample size: 1.0 ml. Fractions were pooled as indicated. (b) SDS-PAGE of pooled fractions, non-reducing conditions, silver staining. Aliquots of the pooled ZZ–brain IGF fractions were freeze-dried. The pellets obtained were dissolved in a constant volume of sample buffer [2.5% SDS–10 m*M* Tris–HCl–1 m*M* EDTA (pH 8.0)] and heated at 95°C for 5 min. Gel: PhastGel Gradient 8–25. Lanes: M, marker proteins (LMW-kit; Pharmacia); S, start material for gel filtration; 1–6, pools 1–6 from (a). kD = Kilodalton.

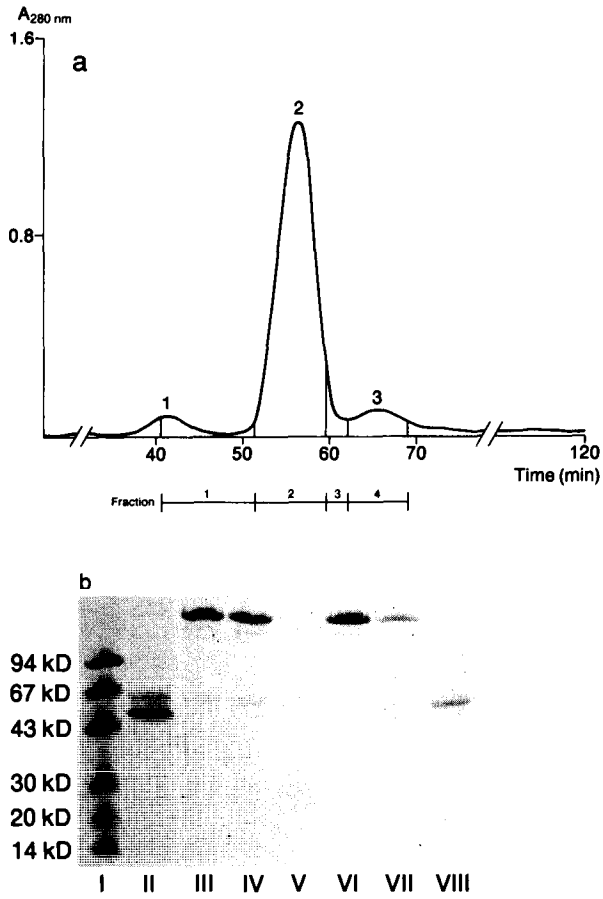


Fig. 17. Final purification of a IgG_{2a} mouse monoclonal antibody on Superdex 200 prep grade. (a) Chromatography. Column: 600 × 60 mm I.D. Feed material: hybridoma cell culture supernatant concentrated by ultrafiltration, clarified by centrifugation and 0.22-μm filtration. Desalted on Sephadex G-25 Coarse. Initial purification by cation exchange using BioPilot Column S Sepharose High Performance. Feed concentration: 11.37 mg total protein/ml. Feed volume: 50 ml (3% of V_c). Buffer: 50 m*M* PBS–0.05% sodium azide (pH 7.5). Flow-rate: 30 cm/h (14 ml/min). Fractions were collected and pooled as indicated. (b) SDS-PAGE of pooled fractions, non-reducing conditions, Commassie brilliant blue staining. Gel: PhastGel Gradient 8–25. Lanes: I, LMW calibration kit; II, flow-through fraction in the preceding ion-exchange step; III and IV, monoclonal antibody from ion-exchange step; V–VIII, fractions 1–4 from Superdex 200 prep grade. kD = Kilodalton.

DISCUSSION

High selectivity in gel filtration is, under ideal conditions, reflected in a steep K_D *versus* log MW curve. The selectivity depends entirely on the properties of the chromatographic support and cannot be modified by adjustment of the eluent composition as in many other chromatographic techniques [3]. The selectivity of macroreticular gels such as agarose gels can be increased by the introduction of

increasing amounts of solid material (agarose), which also leads to a lower exclusion limit. It also inevitably causes the so-called permeability, V_p/V_0, of the column to decrease, which has a clear negative effect on resolution [3,20]. Thus, to reach the exclusion limit of, for example, Sephadex G-200 by increasing the amount of solid material in any macroreticular gel will give a gel with high selectivity as measured by the K_D *versus* log MW relationship but the permeability will decrease dramatically and therefore the resolution will be low.

NMR studies have shown that the polymer chains in a microreticular gel such as Sephadex G-200 retain their mobility to a large extent [21,22]. The ^{13}C NMR spectrum of Superdex 75 prep grade is similar to that of a dextran solution with respect to peak width and resonance frequency (results not shown). Theoretically, the apparent relative support volume, $V_{s,rel}$, as determined with deuterium oxide, would be zero (*i.e.*, $V_{p,rel} = 100\%$) in a gel with complete freedom of movement of the polymer chains constituting the gel, as a small molecule such as deuterium oxide will be able to diffuse to any position in the gel particle. Sephadex G-75 was found in our experiments to have a $V_{s,rel}$ value in the range 1–2%, which is much lower than would be expected for a macroreticular gel with the same content of dry material (the "water regain" of Sephadex G-75 is *ca.* 7.5 ml/g dry material).

By "immobilizing" a dextran solution in the pores of the macroreticular cross-linked agarose gel, we have been able to lower the exclusion limit substantially (from *ca.* 10^7 in the cross-linked agarose gel) without substantially decreasing the permeability. We have thus been able to make rigid, low-exclusion-limit gels with retained high resolution in gel filtration.

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