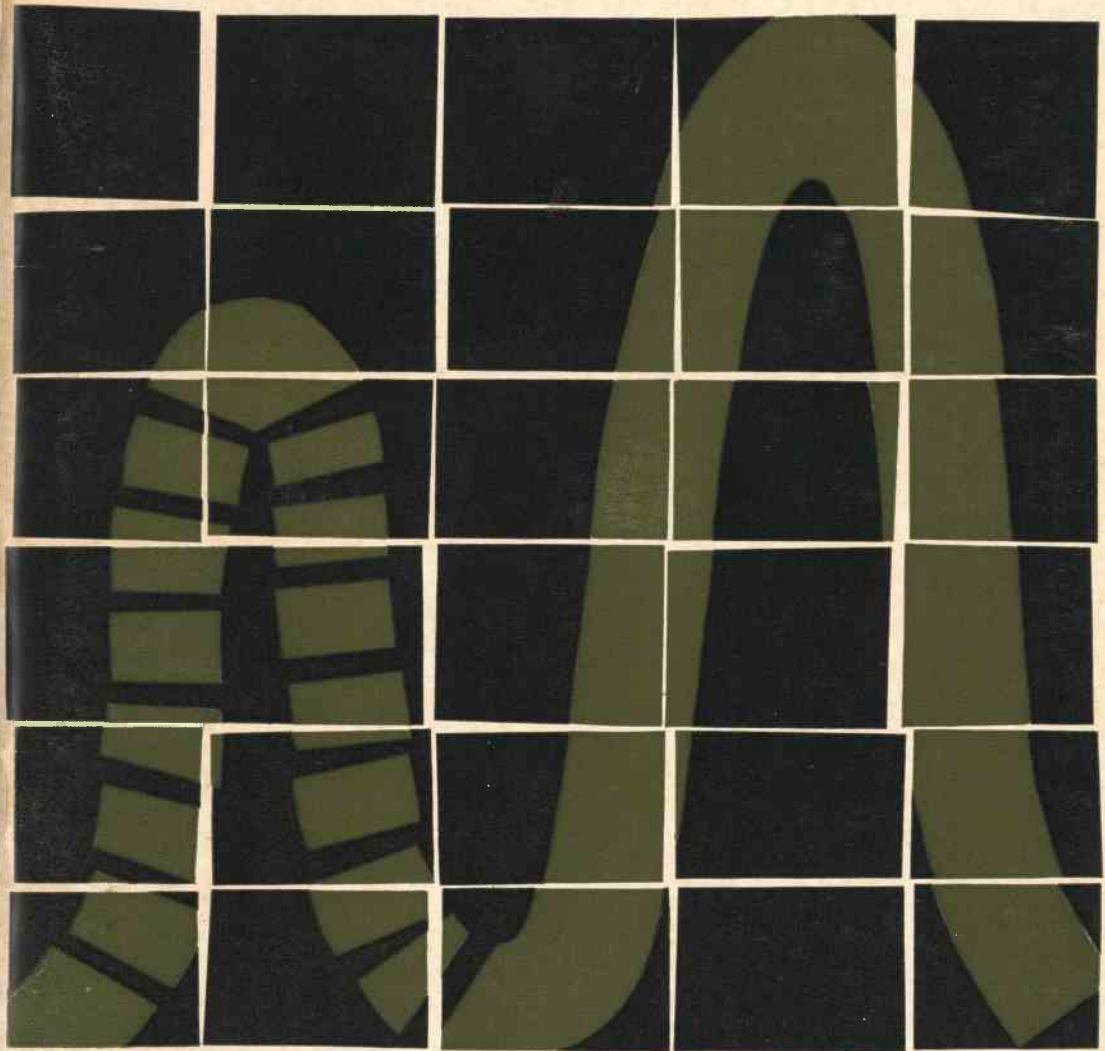


Sephadex[®] — gel filtration in theory and practice *C.F. Bernard*



Pharmacia Fine Chemicals AB is a member of Pharmacia AB, Uppsala, Sweden, the oldest pharmaceutical company in Scandinavia.

Pharmacia pioneered extensive research work on dextran. The plasma volume expander, Macrodex, and the "flow improver", Rheomacrodex, are well known dextran products in hospitals all over the world. Many fine chemicals are now derived from dextran. The most important one, Sephadex, is used for gel filtration. Pharmacia Fine Chemicals also manufactures ion exchangers derived from dextrans, dextran fractions and derivatives and Ficoll, a polymer of high molecular weight with low intrinsic viscosity.

This booklet is intended as an introduction to gel filtration and also as a reference book for those already working in this field. Special emphasis has therefore been given to the sections dealing with gel filtration technique and practical applications.

Pharmacia Fine Chemicals has also issued introductory booklets on the other products mentioned above. These will be sent upon request. Pharmacia Fine Chemicals will be pleased to answer specific questions concerning applications of these products and to hear about new applications or techniques involving their use. Personal communications or reprints of published work would be welcome.

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WHAT IS SEPHADEX?

Sephadex is a modified dextran. The dextran macromolecules are cross-linked to give a three-dimensional network of polysaccharide chains. Because of its high content of hydroxyl groups, Sephadex is strongly hydrophilic and the Sephadex beads swell considerably in water and electrolyte solutions.

Various types of Sephadex are available, differing in their swelling properties.

The degree of swelling is an important characteristic of the gel. Gels in which the matrix is a minor component are used for fractionation of high molecular weight substances, whereas the compact gels are used for work with low molecular weight compounds.

Sephadex of the LH-series is an alkylated product with lipophilic as well as hydrophilic properties. Sephadex LH therefore swells in many organic solvents as well as in water.

Sephadex is supplied in the form of minute beads. The bead form has considerable advantages, as it imparts good flow and separation properties to chromatographic materials.

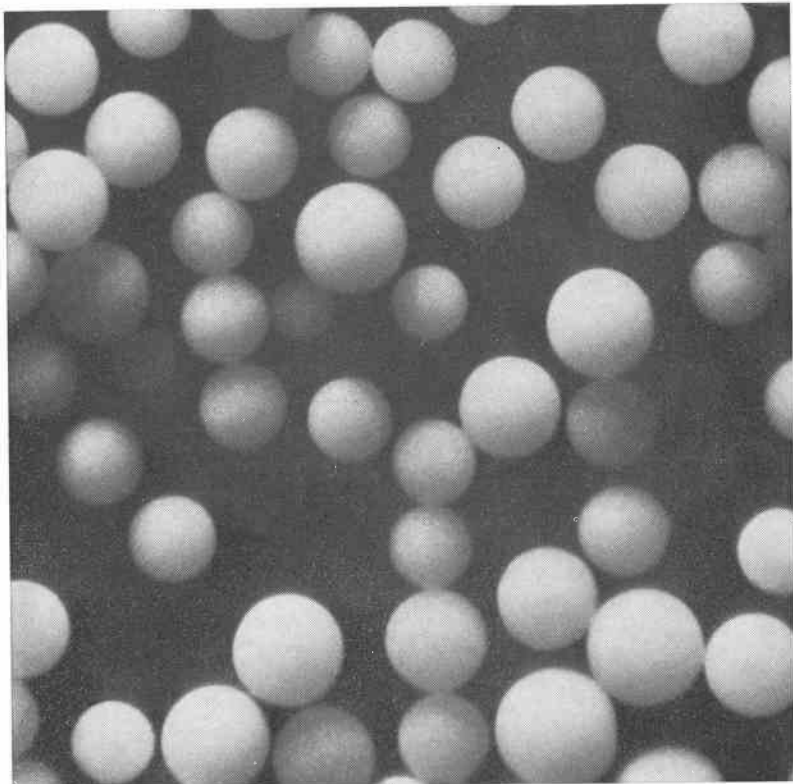


Fig. 1 Bead polymerized Sephadex.

GEL FILTRATION WITH SEPHADEX

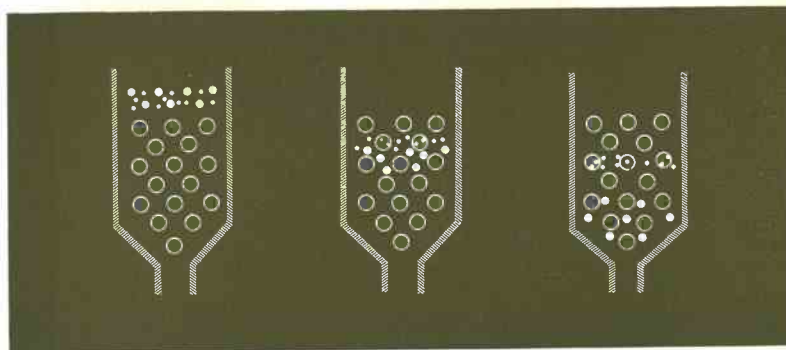
Gel filtration first became an established laboratory technique with the introduction of Sephadex in 1959. Since then, gel filtration has been increasingly used both in analytical and in preparative work, as well as on a production scale in the chemical industry.

Before Sephadex was introduced, fractionation and separation of molecules according to size could only be carried out by very time-consuming or expensive methods. Using the appropriate type and grade of Sephadex, such separations can now be performed rapidly and simply. Sephadex is particularly useful for chromatography of substances of biological origin, which are often very labile.

A gel filtration experiment can be schematically described in the following way.

Molecules larger than the largest pores of the swollen Sephadex, i.e. above the exclusion limit, cannot penetrate the gel particles and therefore they pass through the bed in the liquid phase outside the particles. They are thus eluted first.

Smaller molecules, however, penetrate the gel particles to a varying extent depending on their size and shape. Molecules are therefore eluted from a Sephadex bed in the order of decreasing molecular size.



Three stages of a simple separation on a Sephadex bed are diagrammatically illustrated in the figure. The large dots represent the large molecules, the small dots the small molecules and the open circles Sephadex particles.

As the molecules pass through the bed at different rates, they emerge at the outlet end of the column separated from each other. The zones eluted are somewhat wider than the sample applied, but by the use of suitable experimental conditions this zone broadening can be limited. When all the molecules have been eluted from the Sephadex bed, the column is ready for another experiment. This automatic regeneration is one of the advantages of gel filtration: the same column can in general be used for a large number of experiments.

SEPHADEX PROPERTIES

Physical properties

The properties of Sephadex of the G-series are given in table I. The Sephadex types of the G-series differ in degree of cross-linkage and thus in swelling properties. The water regain value is used to characterize the gels with respect to their swelling ability. It represents the amount of water imbibed by the gel grains on swelling and does not include the water between the grains. The type numbers refer to the water regain value of the gels. Sephadex G-10 thus has a water regain value of 1 and Sephadex G-200 has a water regain value of 20.

Sephadex of the G-series also swell in dimethylsulphoxide, formamide and glycol. Mixtures of water and the lower alcohols may also be used. In addition to these solvents, Sephadex G-10 and G-15 swell in dimethylformamide. It should however be noted that the solvent regain in solvents other than water is not equivalent to the water regain. Sephadex LH-20 is obtained by chemical modification of Sephadex and has the property of swelling in many polar organic solvents as well as in water. The solvent regain values for Sephadex LH-20 are given in table II.

Sephadex is available in different particle size grades. The superfine grade is intended for column chromatography requiring very high resolution and for thin-layer chromatography. The fine grade is recommended for preparative purposes, where the extremely good resolution that can be achieved with the superfine grade is not required, but where the flow rate is of greater importance. The coarse and medium grades are intended for preparative chromatographic processes where a high flow rate at a low operating pressure is essential. In addition the coarse grade is suitable for batch procedures.

Table I

Sephadex type	Particle diameter μ	Water regain ml H ₂ O/g dry Sephadex	Bed volume ml/g dry Sephadex	Fractionation range for	
				peptides and globular proteins	dextrans
Sephadex G-10	40-120	1.0 \pm 0.1	2-3	-	700
Sephadex G-15	40-120	1.5 \pm 0.2	2.5-3.5	-	1,500
Sephadex G-25 Coarse	100-300	2.5 \pm 0.2	4-6	1,000-	5,000
Sephadex G-25 Medium	50-150	2.5 \pm 0.2	4-6	1,000-	5,000
Sephadex G-25 Fine	20-80	2.5 \pm 0.2	4-6	1,000-	5,000
Sephadex G-25 Superfine	10-40	2.5 \pm 0.2	4-6	1,000-	5,000
Sephadex G-50 Coarse	100-300	5.0 \pm 0.3	9-11	1,500-	30,000
Sephadex G-50 Medium	50-150	5.0 \pm 0.3	9-11	1,500-	30,000
Sephadex G-50 Fine	20-80	5.0 \pm 0.3	9-11	1,500-	30,000
Sephadex G-50 Superfine	10-40	5.0 \pm 0.3	9-11	1,500-	30,000
Sephadex G-75	40-120	7.5 \pm 0.5	12-15	3,000-	70,000
Sephadex G-75 Superfine	10-40	7.5 \pm 0.5	12-15	3,000-	70,000
Sephadex G-100	40-120	10.0 \pm 1.0	15-20	4,000-	150,000
Sephadex G-100 Superfine	10-40	10.0 \pm 1.0	15-20	4,000-	150,000
Sephadex G-150	40-120	15.0 \pm 1.5	20-30	5,000-	400,000
Sephadex G-150 Superfine	10-40	15.0 \pm 1.5	20-30	5,000-	400,000
Sephadex G-200	40-120	20.0 \pm 2.0	30-40	5,000-	800,000
Sephadex G-200 Superfine	10-40	20.0 \pm 2.0	30-40	5,000-	800,000

Table II

Solvent regain for Sephadex LH-20 in different solvents

Solvent	Appr. solvent regain ml solvent/g dry gel	Appr. bed volume ml/g dry gel
Dimethylformamide	2.2	4.0 - 4.5
Water	2.1	4.0 - 4.5
Methanol	1.9	4.0 - 4.5
Ethanol	1.8	3.5 - 4.5
Chloroform, stabilized by 1 % ethanol	1.8	3.5 - 4.5
Chloroform	1.6	3.0 - 3.5
n-Butanol	1.6	3.0 - 3.5
Dioxane	1.4	3.0 - 3.5
Tetrahydrofurane	1.4	3.0 - 3.5
Acetone	0.8	
Ethyl acetate	0.4	
Toluene	0.2	

Fractionation ranges and chromatographic properties

The choice of the appropriate Sephadex type depends on the molecular size and the chemical properties of the substances to be separated.

Each Sephadex type fractionates mainly within a particular molecular weight range, determined by the degree of swelling of the gel. Molecules of a molecular weight above the upper limit of this range – the exclusion limit – are totally excluded from the gel. Molecules of a molecular weight below the fractionation range are usually eluted at an elution volume approximately equal to the total bed volume.

Table I gives the molecular weight ranges within which fractionation can be achieved by different Sephadex types. In fig. 2 is given the correlation between K_{av} and molecular weight (logarithmic scale) of proteins on various Sephadex types.

Certain molecules deviate strongly from the general elution behaviour described above. Notably, aromatic substances (homocyclic or heterocyclic) show greater affinity to Sephadex gels than to aqueous solutions, and are thus usually retarded to varying extents compared with non-aromatic substances of similar size. In addition to the affinity of aromatic substances to the matrix of Sephadex in aqueous solutions, some special affinities have been observed in work with Sephadex LH-20 in organic solvents. Thus, solutes containing hydroxyl and carboxyl groups are retarded more than would be expected from their molecular size when they are chromatographed on Sephadex LH-20 in chloroform.

The Sephadex matrix contains a small number of carboxyl groups which at low ionic strength cause interaction between charged solutes and the matrix. Thus, positively charged substances may be retarded, resulting in tailing of the elution curve, while negatively charged substances are excluded from the gel phase. These effects can be eliminated by the use of solvents with an ionic strength exceeding 0.02.

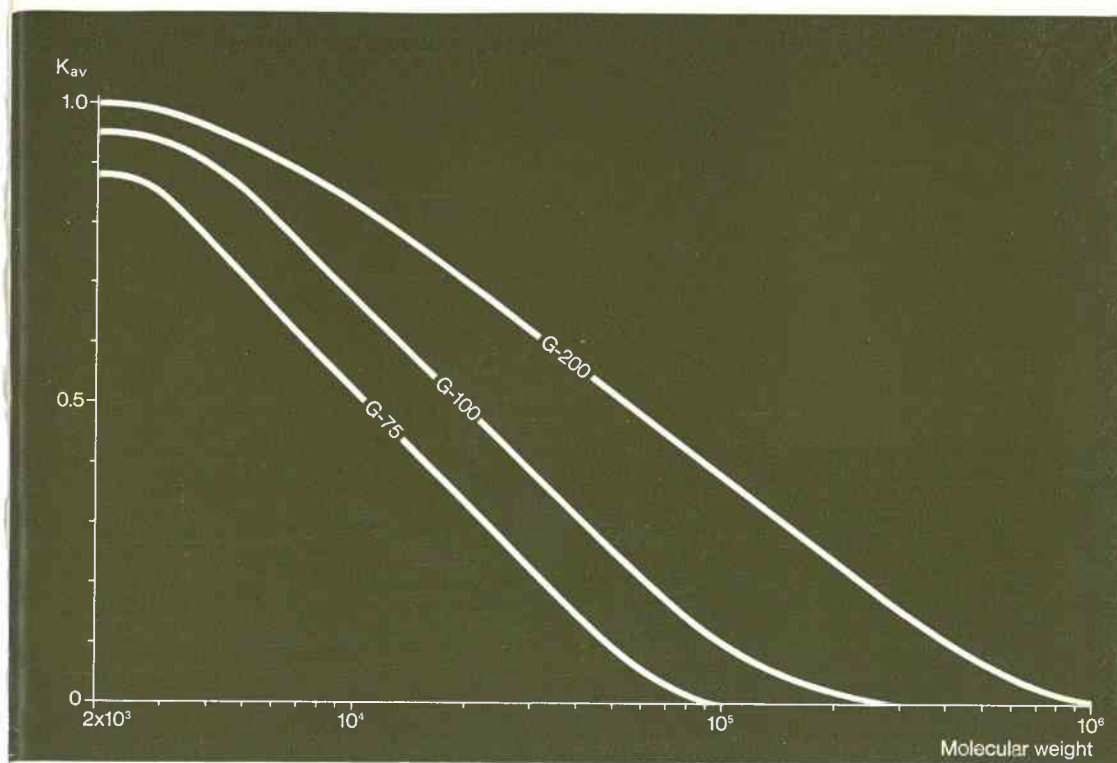


Fig. 2 Relationship between elution behaviour and molecular properties: K_{av} versus molecular weight (on a logarithmic scale) for globular proteins. (By kind permission of the author, P. Andrews.)

Chemical stability of Sephadex

The Sephadex gels are insoluble in all solvents (unless the Sephadex is chemically degraded). They are stable in water, salt solutions, organic solvents, alkaline and weakly acidic solutions. In strong acids the glycosidic linkages in the gel matrix are hydrolysed. However, Sephadex can be exposed to 0.1 M HCl for 1-2 hours without noticeable effects, and in 0.02 M HCl Sephadex is still unaffected after 6 months. Prolonged exposure to oxidizing agents will affect the gel and should be avoided.

Sephadex can be sterilized in the wet state by autoclaving for 40 minutes at 110°C without any changes in the properties of the gel. If dry Sephadex is heated to more than 120°C, it will start to caramelize.

APPLICATIONS

Sephadex for analytical purposes

Analysis of multicomponent samples

In gel filtration quantitative yields are obtained as there is no irreversible retention of small amounts of substance on the columns. This makes Sephadex very useful for analytical purposes.

The chromatographic experiments can be made quantitative by recording continuously some physical variable in the effluent (e.g. light absorption, optical activity, refractive index). The effluent can also be collected in fractions and the amounts of substance in them evaluated by physical, chemical or biological tests.

Gel filtration can often be used for analysis where few or no other methods are available. It is thus used for analysis of protein mixtures and fluids of biological origin. In clinical laboratories, gel filtration is now a standard method for diagnosis of proteinaemias, such as Waldenström's disease.^{1, 2, 3, 4*}

A very common application of gel filtration is the analysis of mixtures of molecules of different molecular weight.⁵ An example of this application is given in fig. 3, showing the separation of raffinose, maltose and glucose from each other and from KCl on Sephadex G-15. Fig. 4 shows the separation of the high molecular weight material, the monomer and the plasticizer in a commercial polystyrene. It should be stressed, however, that the method is not limited to this application. Very often molecules of similar molecular weight can be separated and analysed on Sephadex beds. For instance gel filtration is often used to determine the contents of aromatic substances in a mixture, as aromatic substances are usually retarded on a column compared with aliphatic substances of similar molecular weight (see page 12).⁶

* See References on page 54.

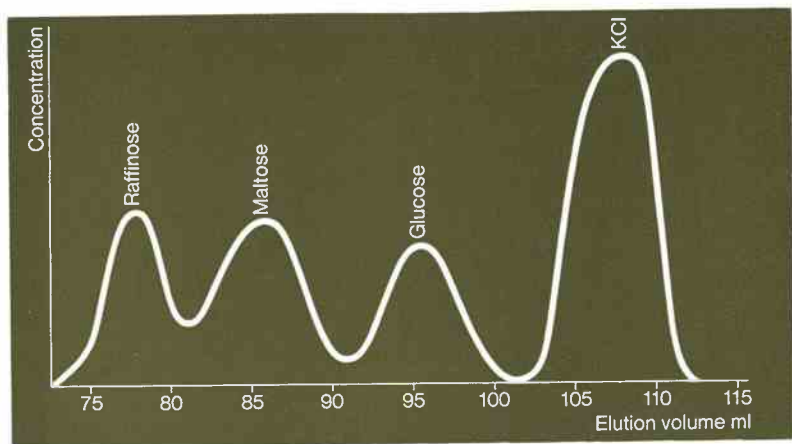


Fig. 3 Desalting and separation of raffinose, maltose and glucose on Sephadex G-15.
 Sample volume: 0.5 ml. Bed dimensions: 1.4×100 cm. Flow rate: 6 ml/h.
 Eluant: 0.025 M phosphate buffer, pH 7.
 Virtually the same result is obtained with distilled water as with phosphate buffer eluant.

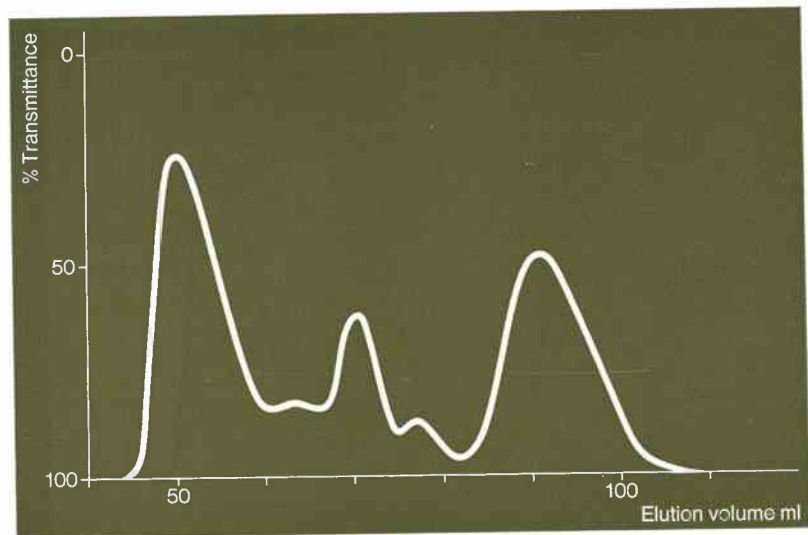


Fig. 4 Elution diagram of a commercial polystyrene (Dow Resin PS-3) from an experiment on Sephadex LH-20.
 Solvent: chloroform. Bed dimensions: 3×20 cm. Sample: 1 ml containing 4 mg of substance. Flow rate: 0.5 ml/min.
 The first peak represents the polystyrene, the second probably corresponds to some plasticizer, while the third is the monomer styrene.

Determination of molecular weights

One of the most striking properties of Sephadex gels as chromatographic materials is their capacity for separating substances according to molecular size. For proteins, extensive investigations have shown that the elution volumes of globular proteins are largely determined by their molecular weight.^{7, 8, 9, 10, 11, 12} Over a considerable range, the elution volume is approximately a linear function of the logarithm of the molecular weight.^{10, 12} This method can also be used for determination of molecular weights of peptides and of other macromolecules than proteins.^{13, 14} As the relationship between molecular weight and elution volume is different for different types of molecules, a separate calibration curve should be determined for each type.

With this method the molecular weight of enzymes can be determined even in crude preparations.^{15, 16, 17} The enzyme activity in the column effluent is measured and thus the elution volume can be determined. From the calibration curve, produced by chromatography of proteins of known molecular weight, the molecular weight of the enzyme can then be estimated. This is the only method of estimating molecular weights of proteins which does not require very extensive purification.

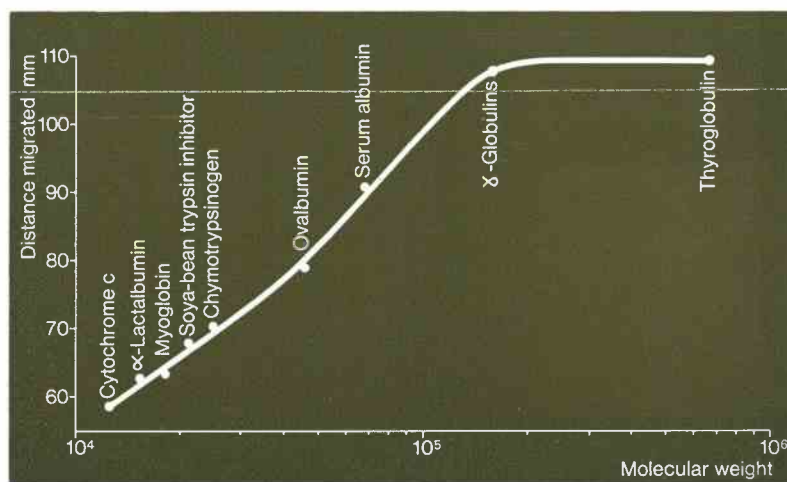


Fig. 5 Relationship between rate of migration and molecular weight (on a logarithmic scale) for globular proteins in thin-layer gel filtration on Sephadex G-100 Superfine. (Andrews, P., *Biochem. J.* 91 (1964) 222—233. Reproduced by kind permission of the Author and the Editor.)

For the determination of molecular weights of small samples of substances, thin-layer gel filtration may be used. The calibration curves in this case are very similar to those obtained in column experiments.^{9, 17}

Fig. 2 illustrates the correlation between elution volume and molecular weight for proteins in column chromatography on Sephadex G-200, while fig. 5 gives the correlation between rate of migration and molecular weight in thin-layer gel filtration on Sephadex G-100 Superfine.

Determination of molecular weight distribution of polymers

The molecular weight distribution is very important for characterization of natural and synthetic polymers. Distribution analysis by classical methods is difficult and tedious as it involves fractionation of the macromolecules by precipitation and determination of molecular weight and amount of substance in every fraction.

The ability of Sephadex columns to fractionate molecules according to molecular weight offers an improved method for distribution analysis of many polymers. The elution curve can be recorded continuously or determined by investigating individual fractions. It is unnecessary to determine molecular weights of the polymers in the effluent fractions of individual experiments, as the chromatographic behaviour of the columns is very reproducible. A calibration curve once determined for the column can be applied to a large number of runs. The precision of this method of distribution analysis is very high due to the good resolution obtained with Sephadex columns. The method has been used for the determination of molecular weight distributions of dextrans, polyvinylpyrrolidones, gelatine preparations and other water-soluble polymers.^{18, 19} In the low molecular weight range this method of distribution analysis can be extended to polymers only soluble in organic solvents by the use of Sephadex LH-20.

Determination of equilibrium constants

In some instances, gel filtration has proved to be a valuable technique for the determination of chemical equilibria. In the case of slow reactions, where the reactants and the products can be separated on a Sephadex column, these substances can be quantitatively determined in the effluent, thereby establishing the position of the equilibrium.^{20, 21, 22}

Gel filtration can also be used to determine the position of equilibria of complex formation where the reactions leading to the equilibria are fast. In this case, one of the reactants is chromatographed in an eluant containing the other reactant. From the elution curve of the reactants the complex formation can be studied. This method is of great value in the study of protein binding of low molecular weight substances such as drugs.²³

Gel filtration has also been applied to the estimation of reaction rates.²⁴

Preparative applications

Desalting

Desalting was one of the first applications of gel filtration to be described, and it is still one of the most important.

In this context the term "desalting" refers not only to the removal of salts, but also to the removal of other low molecular weight compounds from solutions of macromolecules. Thus, according to this definition, the production of lactose-free milk is considered as a desalting process. In desalting, the high molecular weight substances to be desalted usually move with the void volume, while the low molecular weight components are approximately equally distributed between the stationary phase and the mobile phase. As the separation between the compounds of high and low molecular weight is very good, large sample volumes may be used. This is illustrated in fig. 6, which gives the elution curves from a desalting column at different sample size. Sample volumes up to approximately 30 % of the total bed volume may be used.²⁵

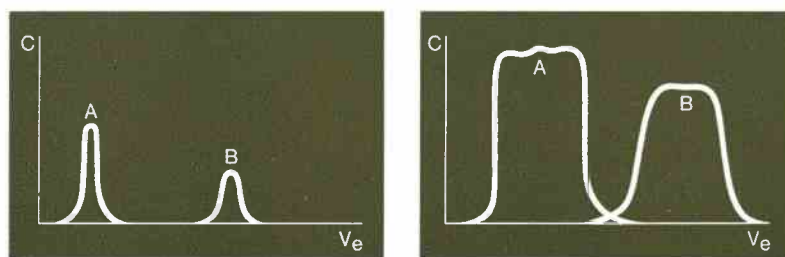


Fig. 6 Elution curves of two experiments on a 300 ml bed of Sephadex G-25. Sample volumes in the two experiments differ in the ratio of 1:10. Bovine serum albumin (A) was separated from NaCl (B) using distilled water as eluant. Even under the extreme conditions of the second experiment (sample volume 33 % of gel volume) a complete separation was obtained. An added advantage was that the protein solution was diluted to only 1/4, whereas in the first experiment the protein was diluted ten-fold.

This desalting method has numerous applications. It is a faster and more efficient technique than dialysis and it is therefore often used for desalting labile substances of biological origin. After modification of macromolecules, excess reagent may be removed by gel filtration. Thus, in immunofluorescence experiments, gel filtration is used for separation of the labelled proteins from unreacted fluorescent reagents.^{26, 27, 28} In the phenol extraction method of nucleic acid preparation, aqueous solutions of the nucleic acids containing large amounts of phenol are obtained. Gel filtration may be used to separate the nucleic acids from the phenol.^{29, 30} In the preparation of samples for electrophoresis and ion exchange chromatography, buffers may be conveniently exchanged by gel filtration.²⁵

In desalting, the conventional column technique is limited by the fact that the viscosity of the sample relative to the eluant should not exceed 2-3 (see page 39). To desalt concentrated solutions of high viscosity, the use of a centrifuge technique is advantageous. This technique will be described under the heading of Industrial applications.

Isolation and fractionation of substances

In desalting, the molecules of large molecular weight, which are moving with the void volume, are separated from low molecular weight material. In many cases, this simple fractionation is insufficient. The separation method must be made more specific for the substances to be purified. The use of the most suitable gel type, long Sephadex beds and collection of small fractions allow separation of molecules differing very little in size and properties. An example of such a separation is given in fig. 7, showing the separation of serum proteins on a column of Sephadex G-200. Hemoglobin and ceruloplasmin are also added to the serum sample. As can be seen from the figure, fractionation of the proteins is obtained. Although serum proteins cannot be completely separated in this way, very good enrichment of individual proteins results.

To achieve an efficient fractionation of a sample, the gel type should be chosen, so that the substances of interest fall well within its fractionation range. When working with very complex mixtures, e.g. biological fluids, substances often cannot be isolated in a single

operation. In such cases gel filtration on different Sephadex types can be used in conjunction with other separation methods. These other separation methods should be based on parameters other than molecular size. For instance ion exchange chromatography or preparative electrophoresis may be used.³¹ Fig. 8 illustrates the isolation of transferrin and albumin from plasma by gel filtration followed by ion exchange chromatography on DEAE-Sephadex, a weak anion exchanger based on Sephadex.

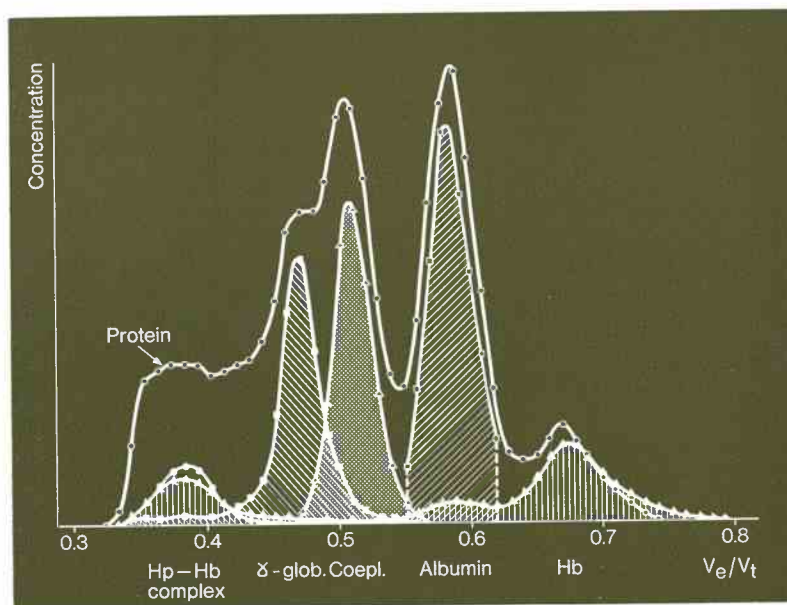


Fig. 7 Fractionation of human serum proteins and hemoglobin on Sephadex G-200. Bed dimensions: 4.2×73.5 cm. Flow rate: 10–20 ml/h. Eluant: 0.1 M tris-HCl buffer, pH 8.0 and 1 M NaCl. (Killander, J., *Biochim. biophys. Acta* 93 (1964) 1–14. Reproduced by kind permission of the Author and the Editor.)

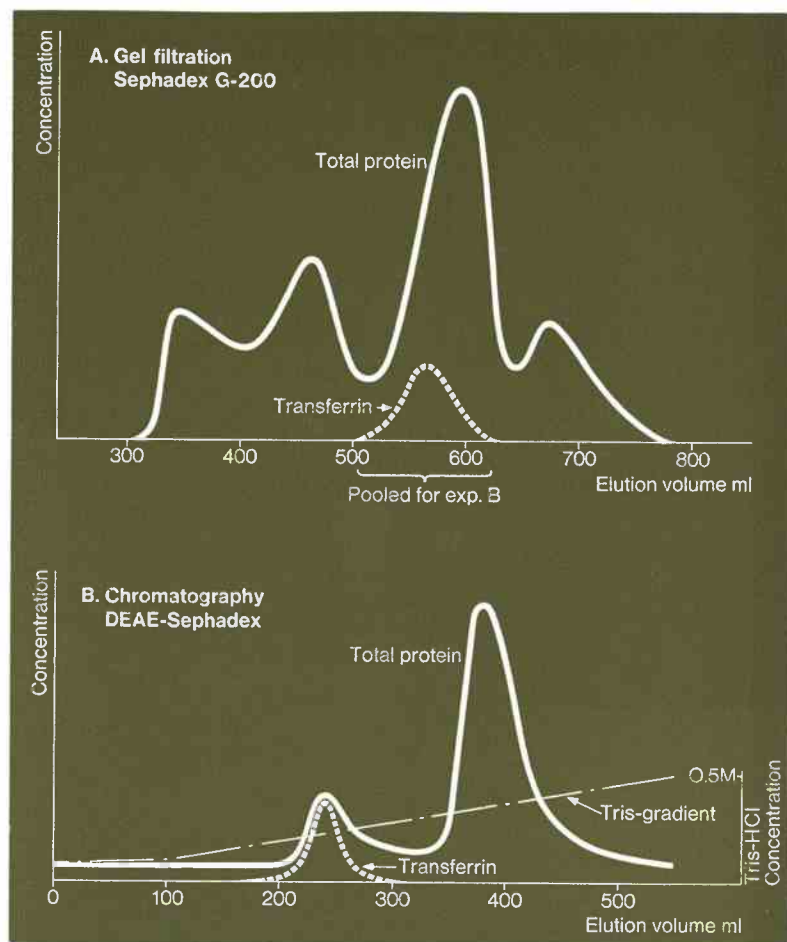


Fig. 8 Purification of transferrin from 6.5 ml lipoprotein-depleted human sera labelled with ^{59}Fe by gel filtration on Sephadex G-200 and chromatography on DEAE-Sephadex A-50.

The gel filtration experiment was performed under the following conditions. Bed dimensions: 4.2×72.5 cm. Flow rate: 20 ml/h. Eluant: 0.1 M tris-HCl buffer, pH 8.0 and 1 M NaCl. The protein content was detected from light absorption at $254 \text{ m}\mu$. The transferrin content was determined from its radioactivity.

The ion exchange experiment was performed on a 2.2×25 cm bed of DEAE-Sephadex A-50. Gradient elution: 0.05 M—0.5 M tris-HCl buffer, pH 8.0. Sample: the third peak from the gel filtration experiment. The transferrin peak from the ion exchanger experiment was homogenous in starch-gel electrophoresis and immunoelectrophoresis. (Reproduced by kind permission of the author, J. Killander.)

In many instances very good purification can be obtained by successive gel filtration experiments under different conditions. Fig. 9 illustrates the purification of vasopressin and oxytocin.^{32, 33} The pituitary extract

used as a starting material contains the peptide hormones complexed to a protein, neurophysin. In the first gel filtration step, the hormones move along with the void volume together with the proteins. In the next experiment the pH is changed so as to dissociate the hormone protein complex. The peptide hormones are now retarded on the bed. This procedure gives very high purification of the hormones.

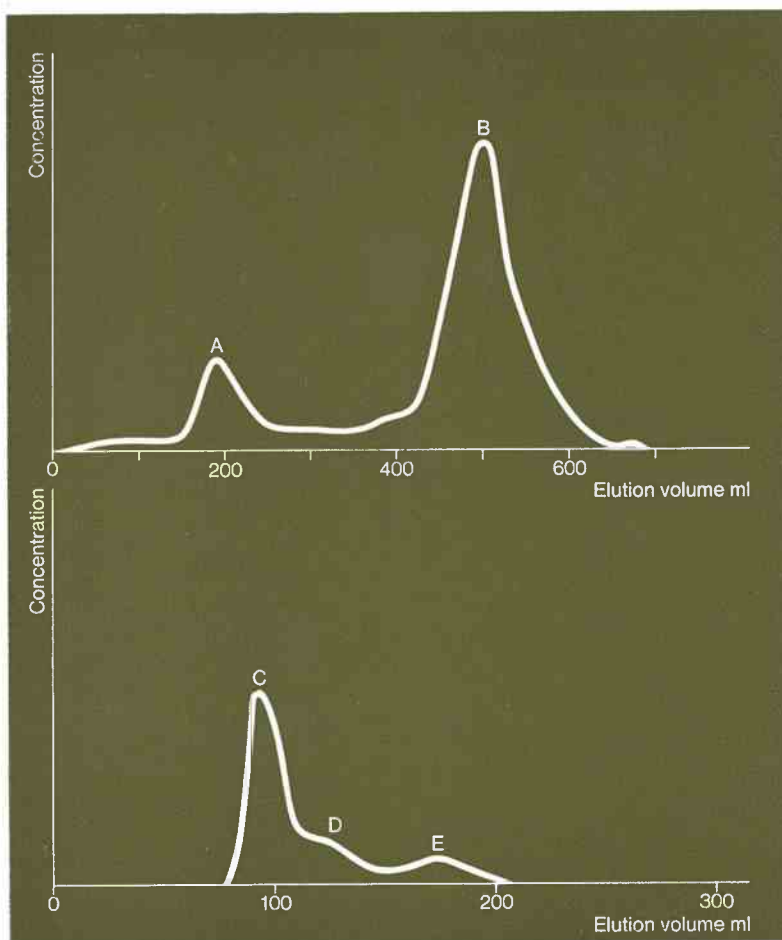


Fig. 9 Purification of oxytocin and vasopressin by gel filtration under varying conditions. In the first experiment, the extract was run on a 4×60 cm Sephadex G-25 bed in 0.2 M pyridine-acetic acid buffer, pH 5.9. The oxytocin and vasopressin are eluted in the void volume (peak A). In the second experiment the same experimental conditions were used except that the eluant was 1 M formic acid. The oxytocin and vasopressin were obtained in peak E. (Lindner, E. B., Elmquist, A., Porath, J., *Nature* 184 (1959) 1565—1566. Reproduced by kind permission of the Authors and the Editor.)

Industrial applications

Sephadex gel filtration can conveniently be applied to isolation and purification processes on a large scale. In many biochemical and pharmaceutical industries extensive development work is in progress to replace by gel filtration previous separation procedures which may have been time-consuming and inefficient. In these applications, the favourable mechanical properties of the dense gels are of great advantage. No regeneration is necessary and very concentrated solutions can be treated.

Some examples of separations on Sephadex are given below.

Serum fractionation

Gel type	Sephadex G-100	
Bed dimensions	diameter:	20 cm
	height:	100 cm
	volume:	32 l
Charge solution	composition:	horse serum
	volume:	0.6 l/cycle
Eluant	0.1 M tris-HCl + 1 M NaCl, pH 8.0	
Flow rate	2.7 l/h	
Elution behaviour	see fig. 10	
Capacity	5 l treated serum/24 h	

For large scale work with Sephadex G-75-G-200, specially designed columns are necessary.

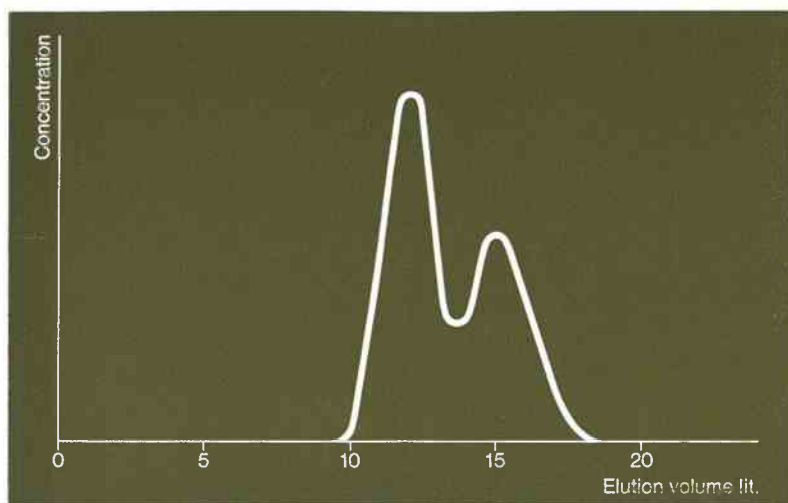


Fig. 10 Fractionation of horse serum proteins on Sephadex G-100 on a production scale. The column used in the experiment permits treatment of 5 l serum/24 hours. The first peak in the elution diagram contains macroglobulins, γ -globulins, haptoglobins etc. The second peak mainly contains the serum albumin, with slight admixtures of other proteins (transferrin etc.). For detailed information on the experiment, see text on page 25.

Desalting

Desalting on Sephadex is a very efficient process, as large columns can be used at a high flow rate. For industrial desalting, Sephadex G-25 Coarse is recommended on account of its good flow properties.

The following results can be obtained in the production of low-lactose milk:

Gel type		Sephadex G-25 Coarse
Bed dimensions	diameter:	45 cm
	height:	60 cm
	volume:	96 l
Charge solution	composition:	skimmilk
	volume:	30 l/cycle
	relative viscosity:	1.5-1.7
Eluant		deionized water
Protein fraction	volume:	45 l/cycle
	recovery of protein:	90 % of input
	recovery of low molecular weight material:	20 % of input
Low molecular weight fraction	volume:	75 l/cycle
	recovery of protein:	10 % of input
	recovery of low molecular weight material:	80 % of input
Flow rate		4 l/min
Cycle time		30 min
Capacity		1500 l treated milk/24 h

To obtain a continuous process, an automated multi-column arrangement may be used. In this type of setup, a number of columns are run with a phase difference between the cycles of the individual columns. This arrangement allows feed and elution pumps to be run uninterruptedly. It is also usually easier to pack and to operate a number of small columns than to use a larger one of the same capacity. In the above example, a setup of four columns would give a capacity of 6000 l/24 h.

Centrifuge desalting

A very promising method for desalting and separation on a preparative and industrial scale is the basket centrifuge technique. This method has two major advantages: it permits the treatment of viscous solutions and gives practically no dilution.³⁴

The principle of the procedure is as follows:

A basket centrifuge is lined with a material which is permeable to liquids yet retains the Sephadex beads. A Sephadex slurry is poured into the centrifuge at rest and the speed of the centrifuge is then raised to $1000\times g$. It is kept at that speed until no more water is spun out, that is until the void volume has left the cake. The Sephadex at this stage forms an even layer around the surface of the basket. The speed is lowered to $60\times g$ and a sample with a volume of 15–20 % of the volume of the Sephadex layer is applied. The speed of the centrifuge is kept at $60\times g$ for about 10 minutes, while the sample progresses through the Sephadex layer. The speed is then increased to $1000\times g$ and kept there until no more solution leaves the centrifuge. The sample, freed from low molecular weight constituents is recovered almost quantitatively. The low molecular weight substances retained in the Sephadex layer can be rinsed out with water at $60\times g$ and the desalting procedure may now be repeated. With coarse grade Sephadex, the retained salt is best washed out with the centrifuge at rest.

For Sephadex G-25, the best results are obtained with the following grades:

Relative sample viscosity (20°C)	Grade
1– 50	Superfine
5– 200	Fine
200–2000	Coarse

Example of centrifuge desalting

Charge solution	volume:	0.50 l
	composition:	191 g Dextran T 250/l 48.8 g NaCl/l
	viscosity:	90 cps (20°C)
Output solution	volume:	0.54 l
	composition:	161 g Dextran T 250/l 0.07 g NaCl/l
Recovery of dextran		91 %
Desalting efficiency		99.8 %
Cycle time		about 2 h
Capacity		about 0.7 kg desalted dextran/24 h

Other applications

Concentration

High molecular weight substances can be concentrated by treatment with dry Sephadex G-25 Coarse. Water and low molecular weight substances are absorbed by the swelling Sephadex beads, while the high molecular weight substances remain in the external solution. After ten minutes the gel grains are removed from the mixture either by a centrifuge (basket type centrifuge or filter adaptors in centrifuge tubes) or by filtration under pressure. In this way an almost three-fold concentration of high molecular weight solutes can be obtained while the ionic strength and the pH are unchanged. The process can be repeated to concentrate the solute even further. This procedure is simple and rapid and is especially useful for very labile proteins.^{35, 36}

Partition chromatography with organic solvents

The hydrophilic character and insolubility of Sephadex also make it useful for column partition chromatography, where one phase is an aqueous solution.^{37, 38, 39, 40, 41} The aqueous phase is held in the gel as the stationary phase, while the organic solvent, which is unable to enter the gel, becomes the mobile phase. The advantage of Sephadex is that such a large proportion of the bed volume is effective as a stationary phase.

Partition chromatography on Sephadex beds has been used successfully to fractionate antibiotics, transfer RNA, peptides and amino acids.

When preparing a bed for partition chromatography, the Sephadex should be swollen and packed in the aqueous phase. The aqueous medium in the void volume is then replaced by the organic phase. Lengthy equilibration of the bed with the mobile phase is necessary before sample application.

Zone precipitation

This method is based on the precipitation of a substance which occurs when the concentration of a precipitating agent, e.g. ammonium sulphate, reaches a certain limit.⁴² A decreasing concentration gradient of the precipitating agent is introduced into the column. When the concentration of the precipitating agent applied to the column has reached a sufficiently low level, the sample is introduced followed by a non-precipitating eluant.

The high molecular weight components of the sample move faster than the gradient. When they reach a zone sufficiently concentrated in the precipitating agent they come out of solution. As the experiment proceeds, the precipitated substances redissolve and move through the column at the rate of the gradient continuously precipitating and redissolving.

Other applications of Sephadex based on rapid and reversible precipitation have been described.

Zone electrophoresis

Sephadex can be used in zone electrophoresis as a supporting material in vertical columns as well as in horizontal troughs and on thin-layer plates. In electrophoresis, Sephadex has the advantage of giving sharp and well-defined zones. The material exhibits low electroosmotic flow and negligible protein adsorption and is also very easy to handle.^{31, 43, 44}

EXPERIMENTAL TECHNIQUE

General

Preparation of the gel

To obtain satisfactory flow rates through the gel and good separations, the gel must be prepared carefully. The gel should be allowed to swell in excess solvent and left to stand for at least the period of time given in table III.

For all the gel types, the process of swelling can be accelerated by putting the gel slurry in a boiling water bath.

Table III

Type of Sephadex	Minimum swelling time	
	at room temperature	on boiling water bath
G-10, G-15, G-25, G-50	3 hours	1 hour
G-75	24 hours	3 hours
G-100, G-150, G-200	3 days	5 hours
LH-20	3 hours	—

Air bubbles may be trapped in the gel slurry, causing uneven flow when the beds are packed. This problem is avoided by swelling the slurry on a boiling water bath. Alternatively the slurry may be de-aerated before packing.

Prevention of bacterial growth on Sephadex

Bacterial and fungal growth may interfere seriously with the chromatographic properties of Sephadex. It will also disturb the flow through the bed. The eluant in columns which are not in use should therefore always contain a small amount of some bacteriostatic agent.

The following substances can be used for this purpose:

Sodium azide, 0.02 %

Hibitane® (chlorhexidine) (ICI), 0.002 %

Chloretone, 1-1-1-trichloro-tert-butanol, 0.1 %

Chloroform, (saturation concentration at room temperature)

Chloroform is not recommended as a bacteriostatic agent on Sephadex G-100, G-150 or G-200, as it causes the gel particles to shrink slightly.

Oxidizing substances should not be used as bacteriostatic agents.

Autoclaving of the gel, see page 14.

Storage and drying of Sephadex of the G-series

After use, Sephadex can conveniently be stored in the swollen state (preferably under refrigeration) after addition of a bacteriostatic agent. However, once swollen it can be dried and restored to its original state by the following procedure:

The swollen gel is washed on a Buchner funnel with excess water to remove salt and contaminants. To shrink the gel, alcohol is then added at intervals under gentle suction. When the shrunken Sephadex has been sucked dry, it can be dried at 60°-80°C. Clumps appearing during this process do not matter, as they will disperse during re-swelling of the gel material. A very slow shrinking procedure will prevent clump formation.

Column chromatography

Selection of chromatographic columns

To obtain best results from gel filtration experiments, care in the choice of column equipment is necessary.

For analytical purposes, a column with an internal diameter of approximately 2.5 cm often proves satisfactory. Narrower columns sometimes tend to give slight wall effects. Wider columns may also give very good results in analytical work. Inconveniently large amounts of sample solution may, however, be necessary. It may also be difficult to achieve an even sample application and an even distribution of the flow rate over the column cross sectional area.

For preparative purposes, very large columns can be used. Sephadex has mechanical properties which give stable beds even of very large dimension. Beds of more than 1000 litres have been used in continuous operations with dense gels as bed material.

The height of the gel bed to be used depends on the nature of the separation. For desalting purposes short columns may be used. For instance, gel beds of 50 cm length or less can be used with coarse grade Sephadex material. With fine grade material still shorter beds give satisfactory resolution.

For more critical separations, longer beds should be used. The mixing chambers at the inlet and the outlet should always be kept as small as possible. Many simple columns have large mixing chambers, which may be filled with glass beads to prevent remixing. Bed supports made from coarse sintered glass or porous plastic cannot be recommended because they easily become clogged by small gel particles.

Sephadex Laboratory Columns

For the convenience of customers, Pharmacia Fine Chemicals has developed a series of standard columns for gel filtration. The design is based on many years' experience in the field of gel filtration. They are manufactured from selected construction materials which do not cause destruction of labile biological substances.

A special column has been developed for experiments with organic solvents, because some parts of the ordinary Sephadex columns are not compatible with these solvents.

The columns have the following special features:

- mixing chamber at the outlet of less than 0.1 % of the column volume, minimizes dilution and prevents remixing
- nylon fabric bed support permits easy flow of the liquid as it does not get clogged during the experiments
- columns are easily assembled and taken apart
- most of the columns can be equipped with a flow adaptor for upward flow experiments and automatic or semiautomatic sample application.

Packing of the Sephadex bed

The following instructions refer to the procedure for packing the Sephadex Laboratory Columns, but it should also be possible to apply them to the packing of other columns.

Packing of Sephadex G-75—G-200 beds. Correct packing of a bed is of utmost importance for good performance.

After the column has been mounted vertically, the outlet should be fitted with a narrow piece of tubing of approximately the length of the column (see fig. 11). A small amount of liquid is poured into the column. The outlet is then closed. Air bubbles trapped under the nylon net should be removed. This can be done by sucking them away with a pipette positioned above the nylon net or by forcing liquid back and forth through the net with a syringe attached to the tubing.

The swollen gel is mixed with sufficient solvent to allow air bubbles to rise from the slurry. A properly packed bed must not contain any air bubbles (see page 31). The column is filled with the slurry, preferably by pouring down a glass rod. If possible, all the slurry required to produce the whole bed is poured into the column at one time. To make this possible, an extension tube must usually be mounted on the top of the chromatographic tube to hold the excess slurry. At this stage, the gel slurry may be gently stirred with a glass rod to remove air bubbles.

The outlet tubing is positioned just below the liquid level in the column and opened to allow the solvent to flow out slowly. If all the

gel necessary for packing the gel bed was not poured into the column at the onset, the remaining slurry may now be added as the excess solvent drains out of the column. The gel in the column should never be allowed to settle completely before the addition of more slurry. A slowly rising horizontal surface of gel bed indicates uniform packing. The column outlet tubing is gradually lowered, until the operating pressure to be used in the experiments is reached. This final operating pressure should not exceed 100–150 mm in the case of Sephadex G-200. Somewhat higher pressures can be used for Sephadex G-75, G-100 and G-150.



Fig. 11 Column packing.

Packing of Sephadex G-10—G-50 beds. As beds of these gels are very easy to pack, column packing very rarely causes any difficulties. The method described above may be simplified as follows. As it is not necessary to limit the operating pressure, very long effluent tubings are not required. As the gel slurry can usually be poured into the chromatographic tube in one operation, extension tubes may be dispensed with. Shorter equilibration than is required for more porous gels is sufficient (see page 37).

Packing of Sephadex LH-20 beds. In most solvents the same method can be used for packing beds of Sephadex LH-20 as for packing beds of Sephadex G-10-G-50. In some solvents, notably in chloroform, the LH-20 material is lighter than the solvent and floats in it. A high flow rate should be applied immediately after pouring the slurry in. The use of a column equipped with a flow adaptor is necessary to keep the bed in position after the flow has been stopped. By the application of a very high flow through the column from the very beginning, the gel bed can be forced to pack in the lower part of the column in spite of its tendency to float. The flow adaptor must then be adjusted to the top of the gel bed before the flow is interrupted.

If a column equipped with two flow adaptors is available the following procedure is recommended. Pour all the required slurry in the column and drain until there is sufficient space to insert the second adaptor. After having locked this adaptor in a position some millimetres above the surface of the gel slurry, the chloroform flow is directed upwards. The bed will now be packed against the upper flow adaptor and it can be fixed in that position by slowly pushing the lower adaptor upwards with a rotating movement.

Stabilizing and testing the packing

Before the sample is applied to the bed, the upper surface of the bed should be protected by inserting a sample applicator or a flow adaptor into the column.

In columns not equipped with a sample applicator or a flow adaptor, a circular piece of a suitable stabilizing material, e.g. filter paper, may be placed on top of the bed.

When inserting the sample applicator, care should be taken not to trap any air bubbles under the nylon net. This is difficult when using dirty sample applicators. They should therefore be cleaned thoroughly before use. The best way of introducing the sample applicator is to wet the net and then to remove excess liquid before insertion.

When a flow adaptor is used, the space above the nylon net should first be filled with solvent. Any air bubbles remaining can then be removed as described previously for the bed support. When the adaptor is introduced into the column, no air bubbles should be trapped under it. To insert the adaptor, fill the whole space above the gel bed

with the solvent and insert the adaptor at an angle. The column outlet must be closed when the flow adaptor is inserted.

To stabilize the bed, the elution medium selected for the experiment should be allowed to run through until at least two or three times the bed volume has passed.

Before starting any experiments, it is advisable to check the homogeneity of the bed by running through a solution of a coloured substance. By watching the progress of the coloured zone through the bed, one can judge the quality of the packing. Blue Dextran 2000, another product of Pharmacia Fine Chemicals, can be used for this purpose.

Sample application

Various methods may be used for sample application on Sephadex beds.

- Most of the eluant above the gel surface is removed by suction. The column outlet is opened and the remaining eluant drained away. After closing the outlet, the sample is carefully pipetted on top of the bed. To prevent disturbance of the surface, a pipette with a bent tip may be used. The column outlet is now opened. After the sample has drained into the bed, the surface (and the column wall in contact with the sample) is washed with a small amount of eluant. The column is then filled with eluant and connected to an eluant reservoir. Under no circumstances should the bed run dry during this procedure.

- With Sephadex G-75, G-100, G-150 and G-200, and particularly in the separation of proteins, it is advisable to keep an eluant layer a few cm deep on top of the bed and to apply the sample beneath this layer. This can be done by means of a syringe or a pipette. The sample must have a greater density than the eluant. If it is not dense enough, a suitable amount of glucose or some other inert low molecular weight compound must be added.

- A very convenient way of applying the sample is to use a flow adaptor. This method is the only possible one for upward flow elution. It is also recommended for downward flow experiments.

Sample size

For analytical purposes, where the main objective is to obtain as good a separation as possible, the starting zone should be as narrow as possible. This is accomplished by using the smallest sample size which gives an even distribution of the sample over the whole bed surface.

With careful sample application, this can be as little as 0.3–0.5 ml for the 15 mm columns and 1–2 ml for the 25 mm columns.

On a preparative scale, where the objective is to obtain a maximum yield with a given degree of separation, the sample should be made as large as possible within the limits set by the requirements of the separation. The volume which separates the front of the elution curve of one substance from that of another substance, that is the difference between their elution volumes, is called the separation volume:

$$V_{\text{sep}} = V'_e - V''_e = V_s(K' - K'') \quad (1)$$

(see page 43).

Were no zone broadening to take place, the whole separation volume could be filled with the substance which is eluted first. The sample

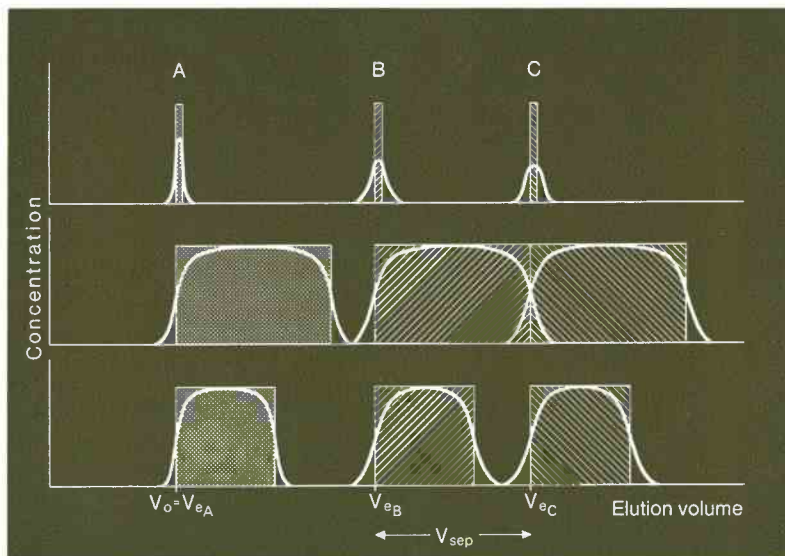


Fig. 12 Elution curves for different sample size. The top diagram corresponds to the application of a small sample. The centre diagram corresponds to the maximum sample size giving complete separation if no zone broadening were to take place. The bottom diagram corresponds to the maximum sample volume to be applied to obtain complete separation in the conditions of the experiment. The shaded areas correspond to the elution profiles that would be obtained if no zone broadening were to take place.

size in this theoretical case could then be as large as the separation volume. However, due to microturbulence, to non-equilibrium between the stationary phase and the mobile phase and to longitudinal diffusion in the bed, the zones will always be broadened. The sample size must therefore be smaller than the separation volume. This is illus-

trated in fig. 12. Fig. 6 gives an example of the elution curves in desalting operations on Sephadex G-25 at different sample volumes. This figure also shows that a larger sample volume gives a smaller dilution.

Sample composition

Due to the linear partition isotherms, gel filtration is remarkably independent of sample concentration. However, in addition to the solubility of the solutes, the viscosity of the sample often limits the concentration that can be used. A high viscosity of the sample causes

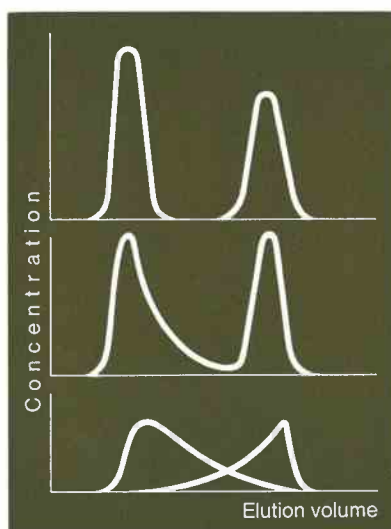


Fig. 13 Elution diagrams obtained when hemoglobin and NaCl were separated. Experimental conditions were identical except that the viscosities were altered by the addition of increasing amounts of dextran. An increasing deterioration of the separation becomes strikingly apparent. (A lower flow rate will not improve the separation.)

Experiment	Dextran T 250 %	η rel
A	0	1.0
B	2.5	4.2
C	5.0	11.8

instability of the zone and an irregular flow pattern. This leads to very broad and skew zones. The critical variable is the viscosity of the sample relative to the eluant. This is illustrated in fig. 13, giving the elution profiles of hemoglobin and NaCl at different sample viscosities. The zones corresponding to low values of K are less broadened than zones corresponding to high K -values (for definition of K -values, see page 48). The reason for this is that at low K -values the attainment of equilibrium between the gel phase and the mobile phase for a given zone sharpness is less critical than for the substances with higher K -values. This is a general phenomenon in chromatography.

Eluant

Completely uncharged substances can be eluted with distilled water. When chromatographing substances carrying charged groups, an eluant with an ionic strength of at least 0.02 may give better results, as Sephadex contains a small number of carboxyl groups giving rise to an adsorption of small amounts of cations and an exclusion of small amounts of anions. If the product is to be lyophilized, a volatile buffer such as ammonium bicarbonate or ammonium formate can be used. In desalting, the separation volume is so large that in general charged substances can also be treated with distilled water as eluant. A complete removal of salt is not possible, but the amount of ions excluded, and therefore eluted in the high molecular weight fraction, is so small that it can be neglected in most cases.

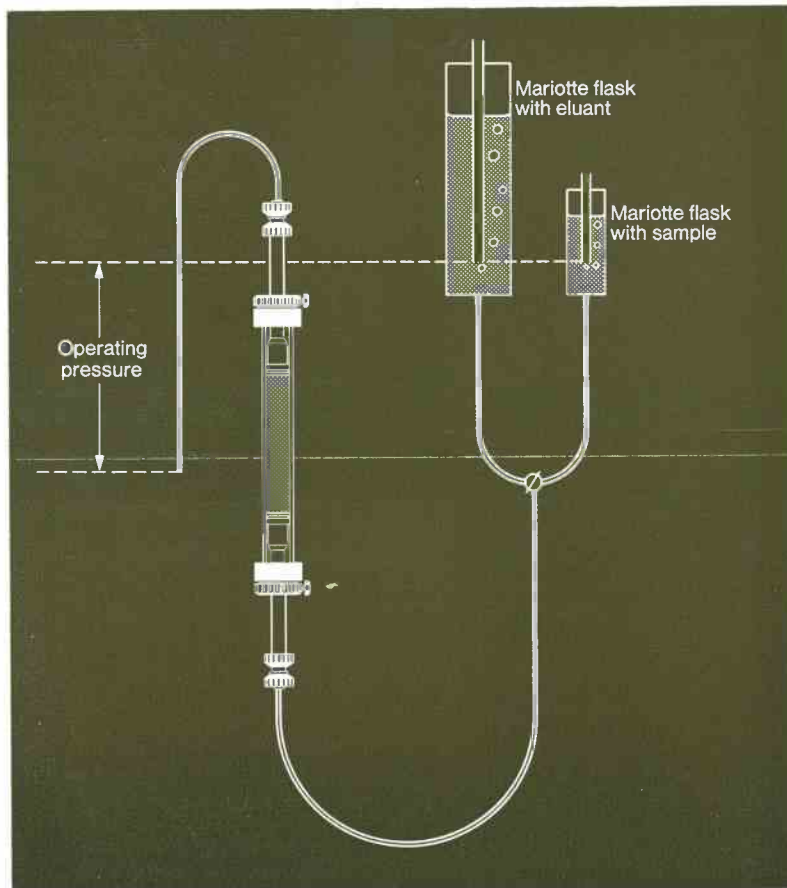


Fig. 14 Experimental setup in gel filtration.

Elution

The flow of liquid in the elution step can be produced by difference in hydrostatic pressure or by a pump.

When the flow is maintained by gravity feed, the use of a constant pressure flask, such as a Mariotte flask, is recommended. Note that in the case of a Mariotte flask, the operating pressure corresponds to the vertical distance between the lower end of the flask air inlet tube and the free end of the column outlet tubing. The experimental setup with Mariotte flasks attached is illustrated in fig. 14.

Influence of flow rate and particle size on zone broadening

High flow rates and coarse grade bed material invariably give large zone broadening, especially in small columns. For analytical purposes the superfine or fine grade material should thus be used, and the flow rate should be kept as low as convenience permits.

For preparative purposes the use of a higher flow rate (and consequently a faster separation) often outweighs the loss of resolution in the chromatographic run. The influence of flow rate and particle size is illustrated in fig. 15 and 16.

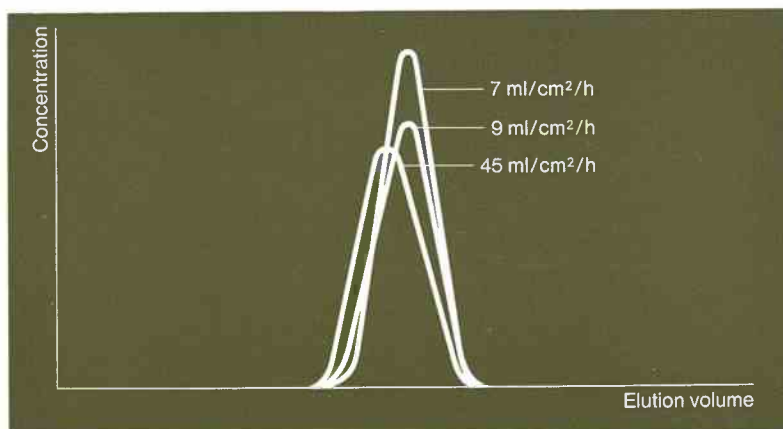


Fig. 15 Elution diagrams of glucose on Sephadex G-25 at different flow rates. A slight displacement of the peak is observed, indicating that a complete diffusion equilibrium has not been reached.

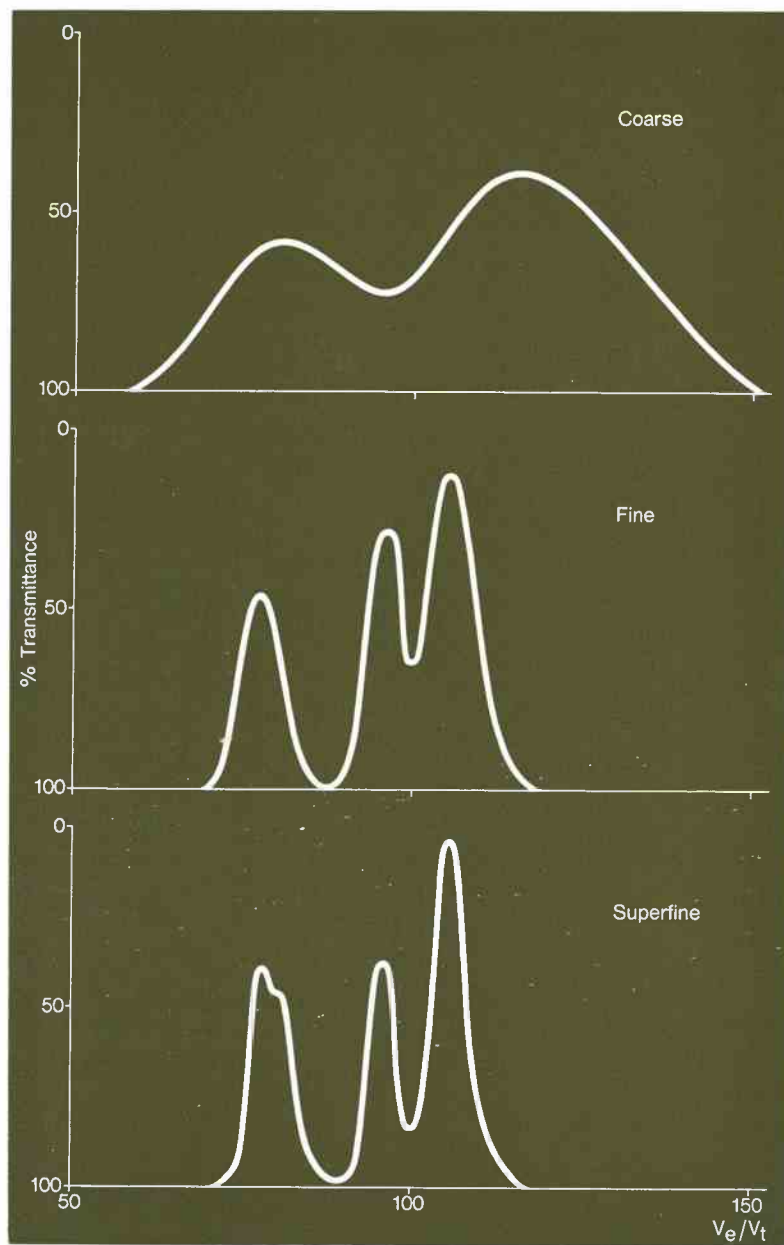


Fig. 16 Gel filtration of cytidine, cytosine and cytidylic acid on Sephadex G-25 of different grades. On the coarse grade only cytidine and cytidylic acid were chromatographed. Bed diameter: 2.4 cm. Bed volume: appr. 200 ml. Flow rate: appr. 1 ml/min. Eluant: 0.025 M phosphate buffer, pH 7.0.

Increasing effective bed height

If incomplete separation is achieved between two substances due to an insufficient separation volume (see page 38) a longer bed should be used. If this involves inconveniently long beds and impractically low flow rates, the effective bed height can be increased by arranging a battery of several columns. The outlet of the first is attached to the inlet of the next by narrow tubing. The beds should be as long as possible thereby reducing the required number, as the connections between them invariably cause some zone broadening.

An increase in effective length can also be achieved by a technique called recycling chromatography. This involves pumping the effluent from the outlet of the column back to the top. Peaks that are satisfactorily separated in the first cycle, should be eluted, as there is a definite risk that after a number of cycles these peaks will overtake other peaks and remixing will occur. As in battery operation, long beds should be used to decrease zone broadening caused by pumping and to reduce the risk of remixing. The use of long beds also reduces the number of cycles required to achieve complete separation of the substances under study.

Repacking

No special regeneration of the bed after an experiment is necessary. The flow rate may be somewhat reduced after lengthy use, mainly because of compression of the gel bed under the influence of the flow. Original flow rates can be restored by backwashing, repacking or reversal of the flow.

Very often a reduction of the flow rate is caused by dust particles etc., accumulating on top of the gel bed. This can be remedied by stirring up a few centimeters and allowing the gel to settle again or by removing a thin layer from the surface of the bed.

Thin-layer gel filtration

In the last few years the technique of thin-layer chromatography has expanded rapidly, and it is now used in many different fields. The greatest advantages of this chromatographic technique are simplicity, speed and microanalytical character.

For thin-layer gel filtration the superfine grade of Sephadex should be used. The preparation of the material is the same as for column chromatography. Sufficient swelling time should be allowed. After swelling, the gel particles are allowed to settle, preferably over night, and the supernatant is then removed by suction or by decantation. With the gels G-25-G-50, a small amount of liquid is left above the gel surface.

Spreading

The glass plates, which can be of any size compatible with the chromatographic equipment, must be thoroughly cleaned by washing with a detergent and distilled water or by storing the plates in a concentrated solution of sodium carbonate. This should be followed by thorough washing with water before use. Any traces of dirt or fatty materials remaining on the surface of the plates will result in unsatisfactory spreading of the Sephadex gel.

The clean dry plates are then coated by the swollen gel. Any of the commercially available spreading devices may be used. More satisfactory results can be obtained by using a rod, which can be moved back and forth over the glass plate. It is kept at a suitable distance from the plate by collars (e.g. consisting of electrical tape) on its ends or by distance pieces positioned along the edges of the glass plate. The gel adheres instantly to the plates without the need for added fixative. In the literature the use of layers of different thicknesses has been reported. The most common range is 0.3-0.5 mm.

Setup and experimental technique

The plates should be run by descending chromatography in a closed chamber, without previous drying. An example of an experimental

setup is illustrated in fig. 17. Paper and thin-layer electrophoresis apparatus can often be adapted for this purpose. Note that the filter paper wick should be covered to avoid evaporation. The flow rate through the gel is regulated by the angle of the plate to the horizontal. An angle between 10° and 20° has been found to be the most suitable, although other angles have been used.

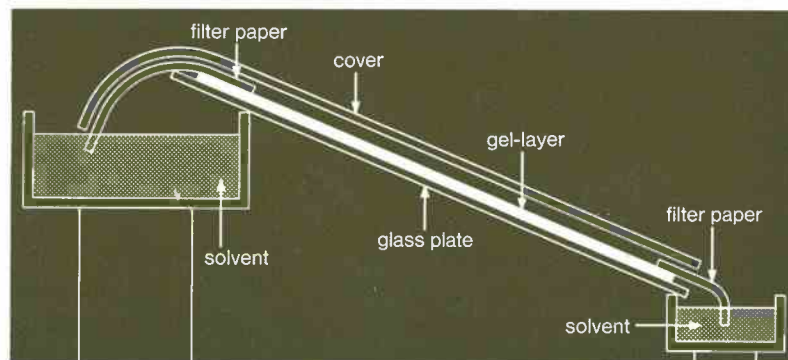


Fig. 17 Experimental setup in thin-layer gel filtration.

Equilibration and sample application. Before a run is started, the plates should be equilibrated by allowing the solvent to flow through the gel for 10 to 15 hours, a step which can easily be carried out overnight. Alternatively the plates can be stored overnight in a saturated atmosphere. After equilibration, the sample ($1-5 \mu\text{l}$) is applied with a micropipette or micrometer syringe as a round spot 2-4 mm in diameter. When the spot is applied, the plate should be horizontal. The spot should be applied with great care, so that the gel layer is not damaged.

Measurement of flow rate. The flow rate through the plate can be measured by the use of a coloured reference substance. Different substances have been used as markers, e.g. DNP-amino acids, coloured proteins such as hemoglobin, cytochrome c, myoglobin or fluorescein isothiocyanate-conjugated γ -globulin. The latter have proved very satisfactory and can easily be detected on the plates by their pronounced greenish yellow fluorescence in ultra violet light.

The best resolution in thin-layer gel filtration is usually obtained at flow rates not exceeding 1-2 cm/hour. An experiment on a 20 cm long plate thus requires no attention for 8-10 hours.

Transfer of spots to filter paper and staining. After completion of a run, the separated substances are transferred from the gel layer by absorption into a superimposed sheet of thick filter paper (e.g. Whatman 3 MM). After about one minute the sheet is removed and can then be dried and stained according to conventional methods. Care should be taken to avoid trapping air between the paper and the gel-coated plate. Extremely small quantities of enzyme can be detected by absorbing the spots into a sheet of paper impregnated with specific substrate.

Alternatively, the spots may be stained directly on the plate after very careful drying of the Sephadex layer. However, this technique cannot be generally recommended because the gel layer has a tendency to crack or pulverize on drying.

Chromatography and electrophoresis. Thin-layer gel filtration can be combined with electrophoresis for better analysis of complex mixtures. Fig. 18 shows a separation of pathological human serum (multiple myeloma serum γ_{1A} -type) on Sephadex G-200 by gel filtration in one dimension followed by electrophoresis in the other dimension.

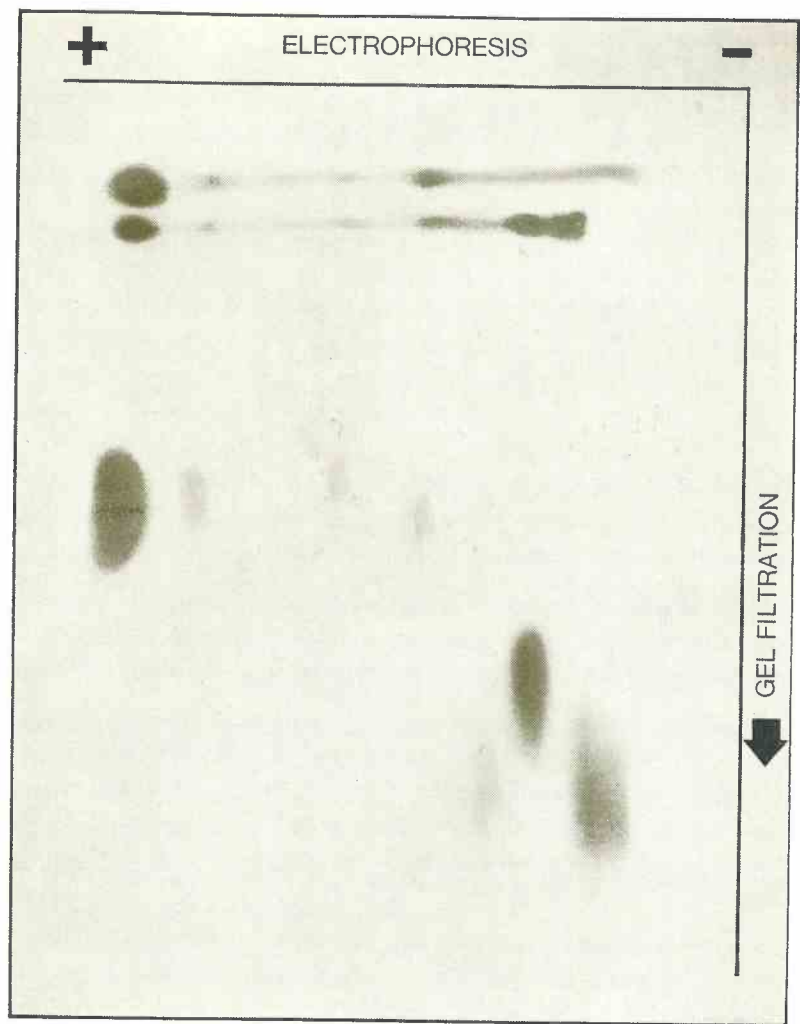


Fig. 18 Gel filtration — electrophoresis.

The figure shows a separation of pathologic human serum (multiple myeloma serum of γ_{1A} type) on Sephadex G-200 by gel filtration in one dimension followed by electrophoresis in the other dimension. Normal serum and pathologic serum were added as references before electrophoresis was started. The electrophoretic separation was carried out in 0.05 M veronal buffer, pH 8.4, with a potential gradient of 6 V/cm for 6 h. (Hanson, L. A., Johansson, B. G., Rymo, L., Clin. chim. Acta 14 (1966) 391—398. Reproduced by kind permission of the Authors.)

CALCULATION OF EXPERIMENTAL RESULTS

To characterize the behaviour of a solute in gel filtration, its elution volume (V_e) is measured. Different criteria are used for the determination of the elution volume.

- When very small samples are applied (small enough to be neglected compared with the elution volume), the position of the maximum of the peak in the elution diagram should be taken as the elution volume.
- When large samples are used (giving a plateau region in the elution curve), the volume eluted from the start of sample application to the inflection point (or the half height) of the rising part of the elution peak should be taken as the elution volume. This criterion is also often somewhat incorrectly applied to samples not giving plateau regions.

More sophisticated criteria may also be used to characterize V_e . These, however, involve such complex mathematical calculations that in practice they are of less value.

To characterize the behaviour of the solute without reference to the geometric dimensions of the column, the following four parameters are most commonly used:

- V_e/V_o
relative elution volume, where V_o , the void volume (or the dead volume), is the elution volume for a substance that is completely excluded from the gel. V_o is identical to the volume of the interstitial liquid between the gel grains in the bed. In some cases the inverse of the relative elution volume, R , the retention constant, is also used for the characterization of substances.

The value of V_e/V_o is easy to calculate. However, it is somewhat dependent on the packing of the column. A high operating pressure resulting in small void volume may sometimes increase the value of this parameter. These variations are, however, of minor importance.

•
$$V_e/V_t$$

where V_t is the total volume of the gel bed.

V_e/V_t is also very easy to determine. The measurement of the total volume of the gel bed is preferably done by calibration of the chromatographic tube. It has the same draw-back as V_e/V_o in being susceptible to variations in the packing of the gel bed, although the variation in V_e/V_t is smaller than that in V_e/V_o .

•
$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad (2)$$

is the partition coefficient between the liquid phase and the gel phase. The parameter is independent of the compaction of the gel bed.

•
$$K_d = \frac{V_e - V_o}{V_i} = \frac{V_e - V_o}{V_t - V_o - m_g v_g} \quad (3), (4)$$

Where V_i is the so called inner volume of the gel, m_g is the weight and v_g is the partial specific volume of the gel matrix, $v_g \cong 0.6$.

This is the parameter which was in most common use in early gel filtration literature.

In the calculation of both K_{av} and K_d use is made of the fundamental equation of partition chromatography,

$$V_e = V_o + K \cdot V_s \quad (5)$$

where K is the partition coefficient between the mobile and the stationary phase and V_s is the volume of the stationary phase. For calculation of K_{av} the whole gel phase is considered to be V_s , whereas for the calculation of K_d only the liquid in the gel phase is taken into account.

THEORETICAL CONSIDERATIONS

Since the theory of chromatography is treated in standard text books, only general features will be outlined here, while those aspects relating specifically to gel filtration will be discussed in slightly more detail.

In chromatography, the solute is carried through the chromatographic bed by the mobile phase. The stationary phase acts to retard the progress of the solute through the bed. Different solutes are retarded to varying extents. In partition chromatography, this retardation is effected by partitioning the solute between the mobile phase and the stationary phase.

The rate at which a solute passes through the bed is proportional to the part of the time, calculated on a statistical basis, that the molecules spend in the mobile phase. This fraction of the time spent in the mobile phase in turn is determined by the ratio of the volumes of the mobile phase and the stationary phase, and by the partition coefficient. The partition coefficient of a solute between the stationary phase and the mobile phase thus governs its chromatographic behaviour, as is evident from the fundamental equation of partition chromatography (5). Various theories have been advanced to account for the value of this partition coefficient.

The first theoretical approach to the specific behaviour of gels in chromatography is that of *Flodin*.⁴⁵ He considers the partition of the solute molecules between the gel particles and the interstitial liquid as an entirely steric effect. He points out that in the immediate environment of the cross-links between the gel chains, the gel matrix occupies a great deal of space. Large molecules cannot penetrate into these regions, while small molecules can approach the cross-links more closely. These steric restrictions, according to Flodin, divide the gel into permitted and forbidden regions. The larger the molecular dimensions of the solute, the greater the proportion of the gel constituting the forbidden region. In the permitted region, the concentration of

solute is assumed to be identical to that in the interstitial liquid. This theory leads to the definition of K_d as the volume available to a given molecular species.

The steric approach to gel filtration has been extended in various ways. According to *Porath*⁴⁶, the molecular weight dependence of the partition coefficients in gel filtration corresponds to that which would be produced by conically shaped pores. *Squire*⁴⁷ has derived an expression for the molecular weight dependence of K_d based on the assumption that the distribution of the pore size would correspond to that produced by a set of cones, cylinders and crevices.

A very interesting approach has been suggested by *Laurent* and *Killander*.¹³ They describe the gel matrix as randomly distributed straight rods of infinite length. From this model they calculate the volume available for molecules represented by spheres of various size.

The sterical approach does not take into consideration other factors influencing the distribution between the gel phase and the mobile phase, such as the difference in affinity of the solute to the solvent and to the gel matrix. Partition coefficients with values higher than would be expected from the theories outlined above are described as "adsorption effects". This terminology has the disadvantage of giving the impression that the substances which show "adsorption effects" have non-linear partition isotherms. However, the narrow symmetric elution curves of even very strongly retarded substances indicate that the partition isotherms for these substances are linear.

Especially for the gels of low water regain, which have a high content of gel matrix, the steric approach is unsatisfactory, because on these gels the effects of differences in affinity (making up for the enthalpy part of the partition coefficient) are often just as great as the steric effects (essentially producing the entropy part of the partition coefficient). This has been shown quite clearly by *Marsden*⁴⁸ who calculated the enthalpy and entropy contribution to the K -values of low molecular weight solutes in work with Sephadex G-10 and G-15.

The treatment of gel filtration as a kind of partition chromatography makes a unified treatment possible both for the substances that behave as one would expect from the sterical theories and for those with a behaviour differing from that. If the K -values are not regarded as fractions of the space in the gel but as partition coefficients, K -values higher than 1 are no longer an anomaly. This is even more justified as the partition isotherms are linear both for substances with high and low K -values.

HOW TO ORDER SEPHADEX

Sephadex is available from Pharmacia subsidiaries or representatives in most countries of the world. You will find the appropriate address on the inside back page of this booklet. If there is no Pharmacia representative in your country, please write directly to Pharmacia Fine Chemicals, Uppsala, Sweden.

Detailed information on the specifications and characteristics of the various Sephadex types and grades can be found in this booklet under the heading "Sephadex types and properties".

In addition to the gel filtration products, Pharmacia Fine Chemicals also markets a range of other separation products, including Sephadex chromatographic columns,

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SEPHADEX LITERATURE AND BIBLIOGRAPHY

As an aid to scientists and technical personnel, Pharmacia Fine Chemicals provides an extensive literature service, including specialized introductory publications, such as this booklet.

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Sephadex LH-20 for gel filtration in organic solvents,
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Bibliography

Important publications on the applications of gel filtration and ion exchangers are abstracted and distributed regularly. A comprehensive reference list on the literature in the field which includes a subject index is also available.

Please ask our local representative or Pharmacia Fine Chemicals, Uppsala, Sweden, for this service.

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