

## Chapter 5 : Other Separation Techniques

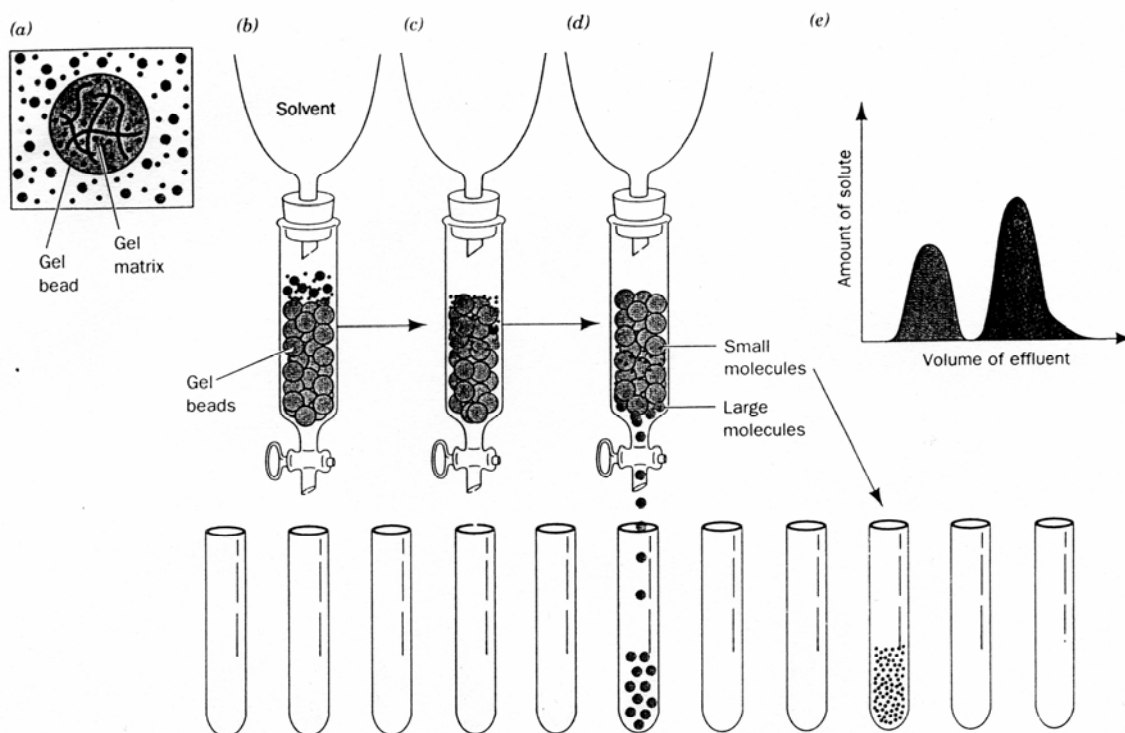
The characteristics of proteins and other biomolecules that are used in the various separation procedures are solubility, ionic charge, molecular size, and binding specificity for other biological molecules.

Characteristic	Procedure
Size	<u>Gel filtration chromatography</u> SDS-PAGE Ultracentrifugation
Charge	<u>Ion exchange chromatography</u> Electrophoresis
Polarity	<u>Hydrophobic interaction chromatography</u>
Binding specificity	<u>Affinity chromatography</u>

## Part 1: Gel Filtration

### I. Introduction

\* In gel filtration molecules in solution are separated according to differences in their sizes as they pass through a column packed with a gel medium. The small molecules which diffuse into the gel beads are delayed in their passage down the column compared with the large molecules which cannot diffuse into the gel and move continuously down the column in the flowing eluent. **The large molecules thus leave the column first followed by the smaller molecules in the order of their sizes.**

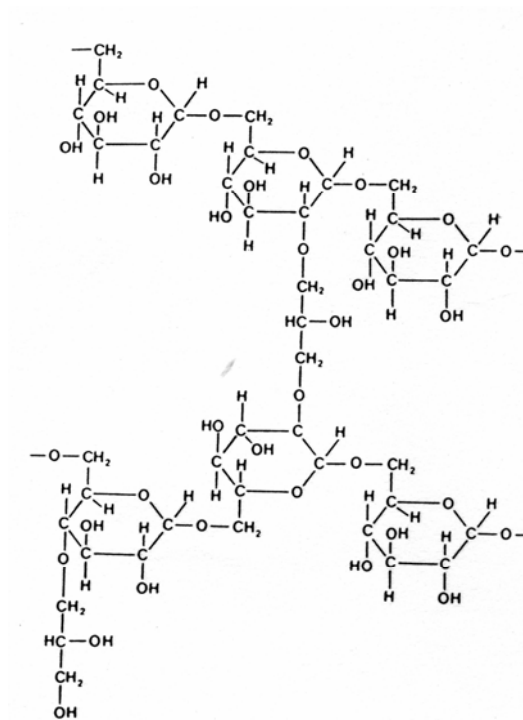


**Figure 5-6. Gel filtration chromatography.** (a) A gel bead consists of a gel matrix (wavy solid lines) that encloses an internal solvent space. Small molecules (red dots) can freely enter the internal space of the gel bead. Large molecules (blue dots) cannot penetrate the gel pores. (b) The sample solution is applied to the top of the column (the gel beads are represented as brown spheres). (c) The small molecules can penetrate the gel and consequently migrate through the column more slowly than the large molecules that are excluded from the gel. (d) The large molecules elute first and are collected as fractions. Small molecules require a larger volume of solvent to elute. (e) The elution diagram, or chromatogram, indicating the complete separation of the two components.

- \* A gel is a heterogeneous phase system in which a continuous liquid phase, usually aqueous, is contained within the pores of a continuous solid phase, the gel matrix. The pores of gels have a carefully controlled range of sizes, and the matrix is chosen for its chemical and physical stability, and inertness (lack of adsorptive properties).
- \* Gels may be formed from polymers by cross-linking to form a three-dimensional network. For example, **Sephadex** is formed by cross-linking dextran. **Composite gels** may be prepared by two kinds of gels. **Superdex** is such a gel. Dextran chains are covalently bonded to a highly cross-linked agarose gel matrix.

### Sephadex:

- \* The medium is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin.
- \* The G-types of Sephadex differ in their degree of cross-linking and hence in their degree of swelling and fractionation range. The Superfine grade is suitable for thin layer gel filtration. The Fine grade is recommended for preparative purposes. 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.



\* Sephadex G-10, G-15, G-25 and G-50 are recommended for separation of **peptides** and other small biomolecules.

Sephadex G-75, G-100, G-150 and G-200 are useful in work with **proteins**.

The DNA grade of Sephadex G-25, G-50, or G-100 should be used for work with DNA or oligonucleotides.

Gel type	Dry bead size $\mu\text{m}$	Fractionation range Globular proteins	Fractionation range Dextran	Swelling factor ml/g
Sephadex G-10	40 – 120	– 700	– 700	2 – 3
Sephadex G-15	40 – 120	– 1 500	– 1 500	2.5 – 3.5
Sephadex G-25 Coarse	100 – 300	1 000 – 5 000	100 – 5 000	4 – 6
Sephadex G-25 Medium	50 – 150	1 000 – 5 000	100 – 5 000	4 – 6
Sephadex G-25 Fine	20 – 80	1 000 – 5 000	100 – 5 000	4 – 6
Sephadex G-25 Superfine	10 – 40	1 000 – 5 000	100 – 5 000	4 – 6
Sephadex G-50 Coarse	100 – 300	1 500 – 30 000	500 – 10 000	9 – 11
Sephadex G-50 Medium	50 – 150	1 500 – 30 000	500 – 10 000	9 – 11
Sephadex G-50 Fine	20 – 80	1 500 – 30 000	500 – 10 000	9 – 11
Sephadex G-50 Superfine	10 – 40	1 500 – 30 000	500 – 10 000	9 – 11
Sephadex G-75	40 – 120	3 000 – 80 000	1 000 – 50 000	12 – 15
Sephadex G-75 Superfine	10 – 40	3 000 – 70 000	1 000 – 50 000	12 – 15
Sephadex G-100	40 – 120	4 000 – 150 000	1 000 – 100 000	15 – 20
Sephadex G-100 Superfine	10 – 40	4 000 – 100 000	1 000 – 100 000	15 – 20
Sephadex G-150	40 – 120	5 000 – 300 000	1 000 – 150 000	20 – 30
Sephadex G-150 Superfine	10 – 40	5 000 – 150 000	1 000 – 150 000	18 – 22
Sephadex G-200	40 – 120	5 000 – 600 000	1 000 – 200 000	30 – 40
Sephadex G-200 Superfine	10 – 40	5 000 – 250 000	1 000 – 150 000	20 – 25

\* Sephadex melts on heating above 40 °C and the bed structure may be irreversibly damaged on freezing. Sephadex cannot be autoclaved, but can be sterilized chemically, for example, by treatment with DEPC (diethylpyrocarbonate).

### Superdex:

\* It is based on highly cross-linked porous agarose beads to which dextran has been covalently bonded.

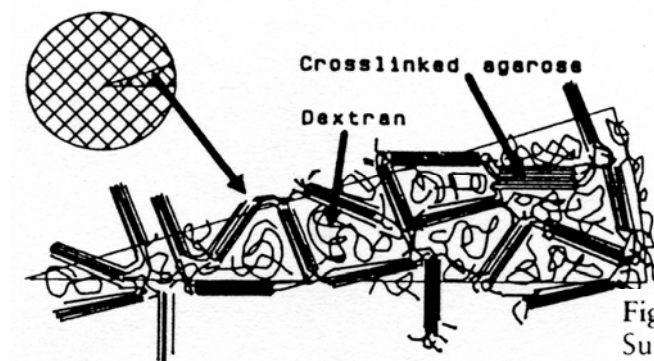


Fig. 15. Structure of Superdex. Dextran chains are covalently linked to a highly cross-linked agarose matrix.

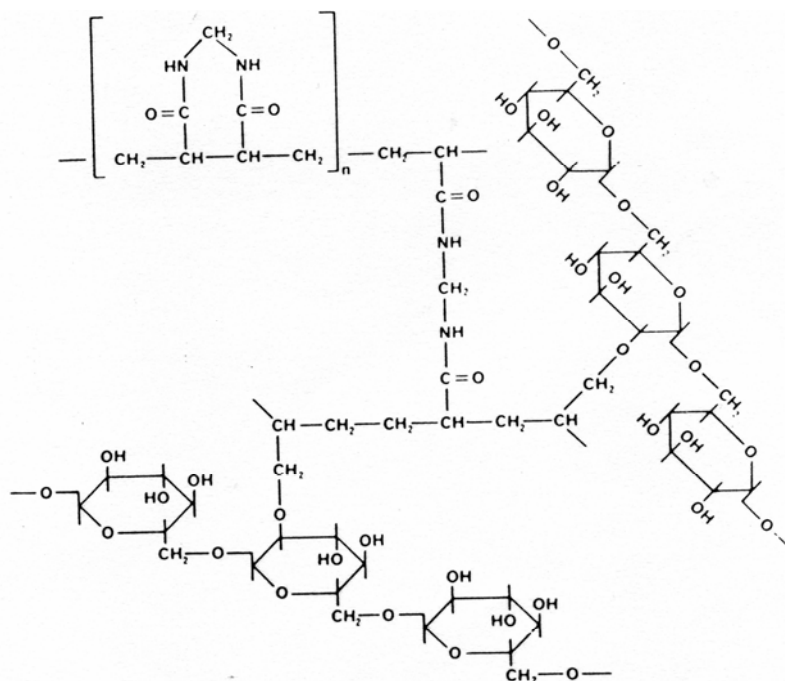
**Table 3. Properties of Superdex**

Gel type	Bead size $\mu\text{m}$	Fractionation range Globular proteins	Fractionation range Dextrans
Superdex peptide	11 – 15	100 – 7 000	–
Superdex 30 prep grade	24 – 44	– 10 000	–
Superdex 75 prep grade	24 – 44	3 000 – 70 000	500 – 30 000
Superdex 75	11 – 15	3 000 – 70 000	500 – 30 000
Superdex 200 prep grade	24 – 44	10 000 – 600 000	1 000 – 100 000
Superdex 200	11 – 15	10 000 – 600 000	1 000 – 100 000

- \* Superdex can be used with all aqueous buffers commonly used in biochemistry with the pH range 3-12, and withstands strong bases and acids.
- \* Superose prep grade may be autoclaved repeatedly at 121 °C, pH 7 for 30 min. without significantly affecting its chromatographic properties.

### **Sephacryl HR:**

- \* Sephacryl High Resolution (HR) is a composite gel prepared by covalently cross-linking dextran with N, N'-methylene bisacrylamide to form a hydrophilic matrix of high mechanical strength.



**Fig. 11. Hypothetical partial structure of Sephacryl HR.**

- \* Sephacryl HR is stable in all aqueous buffers commonly used in biochemistry within the pH range 3-11.
- \* The separation properties of the gel are not affected by detergents e.g. 1% SDS, chaotropic salts or dissociating agents e.g. 8 M urea and 6M guanidine HCl.
- \* Sephacryl HR may be autoclaved repeatedly at 121 °C, pH 7 for 30 min. without significantly affecting its chromatographic properties.

**Table 1.** Properties of Sephacryl HR

Gel type	Bead size µm	Fractionation range Globular proteins	Fractionation range Dextrans	Exclusion limit DNA
Sephacryl S-100 HR	25 – 75	1 000 – 100 000	ND	ND
Sephacryl S-200 HR	25 – 75	5 000 – 250 000	1 000 – 80 000	118
Sephacryl S-300 HR	25 – 75	10 000 – 1 500 000	2 000 – 400 000	118
Sephacryl S-400 HR	25 – 75	20 000 – 8 000 000	10 000 – 2 000 000	271
Sephacryl S-500 HR	25 – 75	ND	40 000 – 20 000 000	1078

### **Sepharose:**

- \* Sepharose is a bead-formed gel prepared from agarose. Although the gel structure of Sepharose is stabilized by H-bonding and not by covalent cross-links, it can be used under most of the conditions encountered in gel filtration.
- \* The agarose used to make Sepharose is obtained by a purification process which removes the charged polysaccharides to give a gel with only a very small number of residual charged groups.
- \* It is stable in water and salt solutions over the pH range 4-9. Chaotropic salts, such as KSCN, should be avoided.

## **II. Experimental Parameters**

- \* **Sample concepts:** A sample volume of 0.5-5% of the bed volume is recommended. Smaller volumes do not normally improve resolution. The **viscosity** of the sample must not be so large as to cause hydrodynamic instability. A high sample viscosity causes instability of the zone and an irregular flow pattern. This leads to very broad and skew zones.
- \* **Column parameters:** The length of the column, cm, is significant since it affects both the resolution and the time taken to elute it.

**Resolution**       $\alpha$       **column length**  
**Elution time**       $\alpha$       **column length**

- \* Eluent parameters: For gel filtration, the pH, ionic strength and composition are not significant as long as they do not affect the sizes or stability of the molecules to be separated.
- \* Running conditions: The lower the flow rate the better the resolution, at least for large molecules.

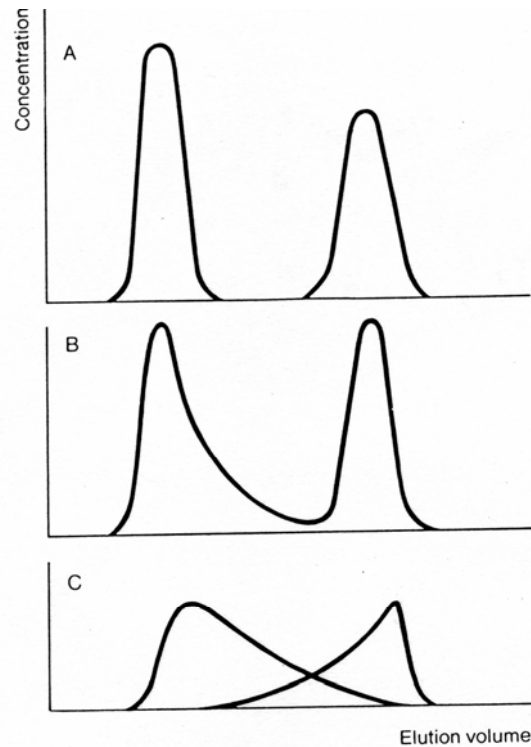


Fig. 33. Elution diagrams obtained when haemoglobin and NaCl were separated. Experimental conditions were identical except that the viscosities were altered by the addition of increasing amounts of dextran. An increasing deterioration of the separation becomes strikingly apparent. (A lower flow rate will not improve the separation.)

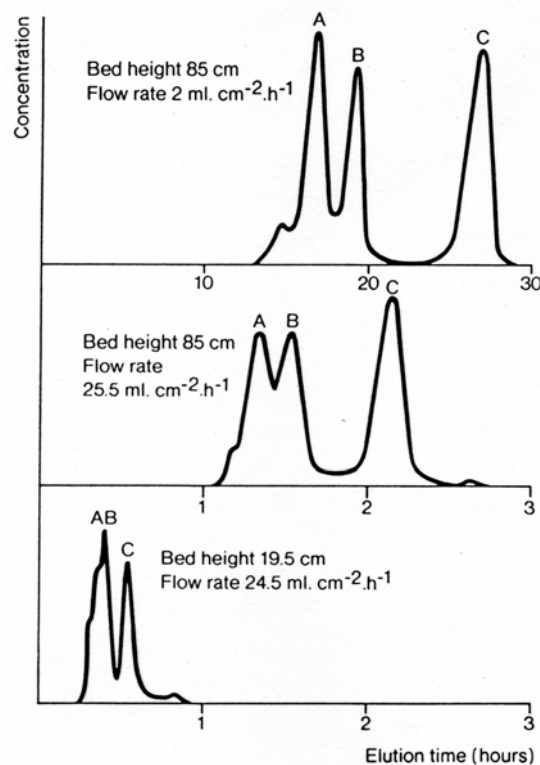
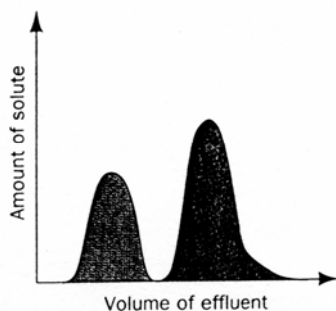


Fig. 34. A model gel filtration experiment to demonstrate optimization of the separation of model proteins A, B and C.

### III. Characterization of Solute Behaviour

- \* Results in gel filtration are typically expressed in the form of an elution diagram showing the variation of solute concentration in the eluent with the volume of eluent passed through the column.



- \* For protein and nucleic acid work and in many other applications continuous detection using a UV-monitor and a recorder gives an immediate permanent record, a chromatogram.

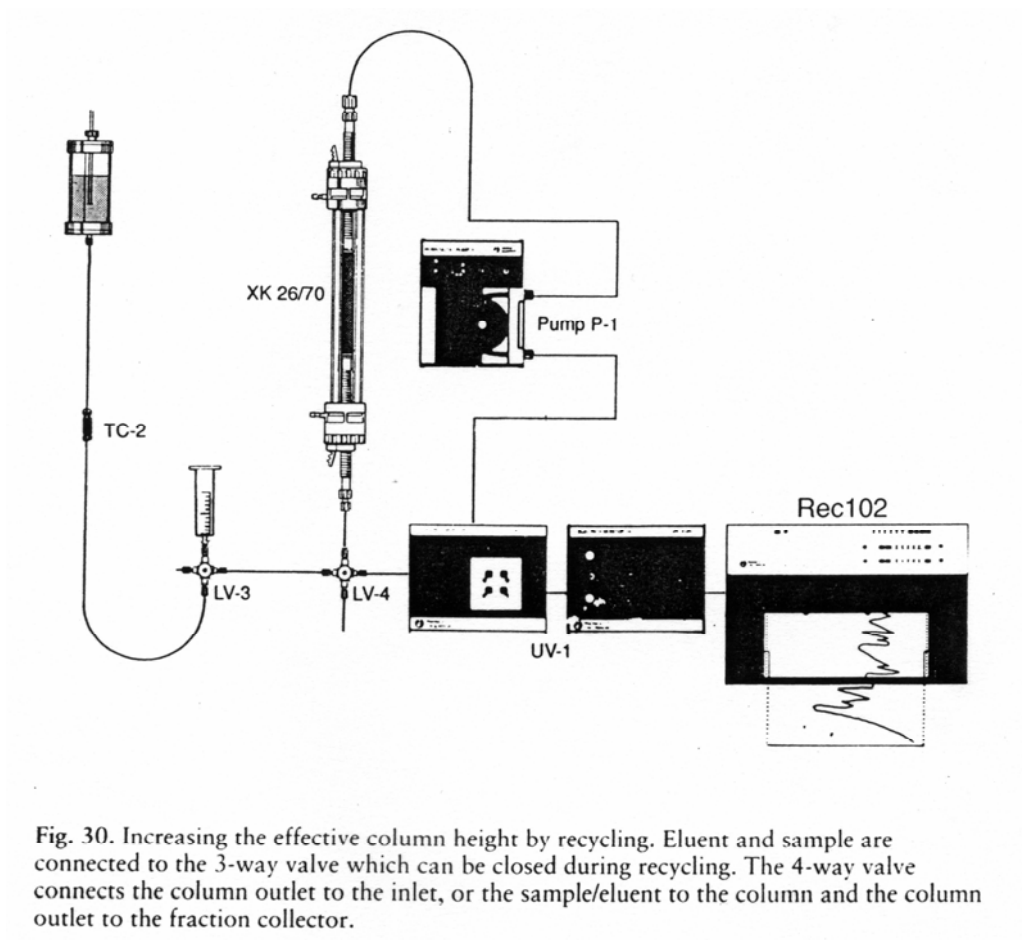


Fig. 30. Increasing the effective column height by recycling. Eluent and sample are connected to the 3-way valve which can be closed during recycling. The 4-way valve connects the column outlet to the inlet, or the sample/eluent to the column and the column outlet to the fraction collector.



## IV. Performing a Gel Filtration Experiment

### 1. Preparing the gel

Pre-swollen media and media which require swelling: Sephadex is supplied as a dry powder and must be allowed to swell in excess solvent before use. During swelling excessive stirring should be avoided as it may break the beads. Do not use magnetic stirrers.

### 2. Packing a column

- 1) The gel suspension should be adjusted so that it is **a fairly thick slurry**. Usually about 75% settled gel is suitable for packing. The gel suspension should reach the temperature of column operation before packing is begun.
- 2) Mount the column on a stable laboratory stand. **Ensure that there are no air bubbles trapped in the dead space under the net** by drawing water through it.
- 3) The gel can be poured directly into the vertically mounted column using a glass rod. If the slurry volume is greater than the volume of the column, a gel reservoir or a column extension should be attached. All the gel required should be poured in a single operation. **Preparing the gel from too thin a suspension or packing the column in stages, often results in a badly packed bed.**

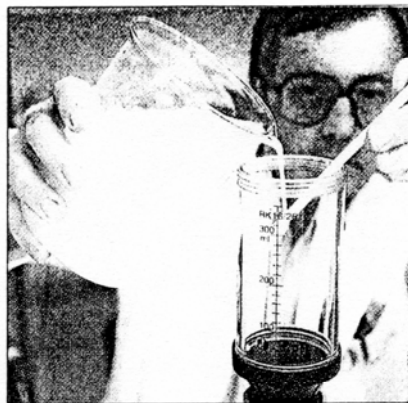


Fig. 38.

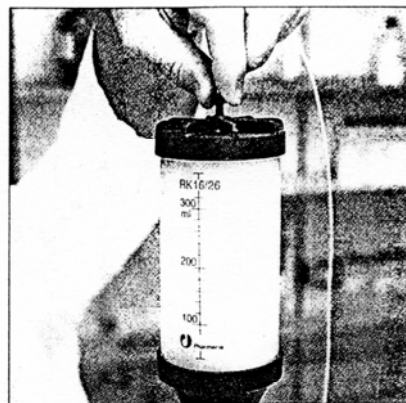


Fig. 39.

- 4) The flow should be started as soon as possible after filling the column to obtain even sedimentation. Two or three column volumes of eluent should be passed through the column in order to stabilize the bed and equilibrate with eluent buffer. A

slightly higher flow rate than is to be used in the experiments should be used for packing.

- 5) Checking the packed bed by running a freshly prepared and filtered solution of a colored substance. Blue Dextran 2000 at a concentration of 2 mg/ml can be used for this purpose. The quality of the packing can be checked by watching the progress of a zone of this substance through the bed. Visual inspection of the bed in transmitted light may also reveal heterogeneities and air bubbles.

### 3. Sample application

Considerable care must be taken to **avoid disturbing the bed surface**. An uneven bed surface leads to uneven separated bands and poor resolution.

- 1) Close the outlet and remove most of the eluent above the gel surface by suction.
- 2) Open the outlet and allow the remaining eluent to drain away. Under no circumstances should the bed be allowed to run dry.
- 3) Layer the sample on top of the bed.
- 4) Open the column outlet and allow the sample to drain into the bed. Do not allow the bed to run dry.
- 5) Wash the sample which remains on the bed surface and on the column wall into the bed with a small amount of eluent.
- 6) Refill the column with eluent and reconnect to a Mariotte flask or pump.

### 4. Cleaning gels and packed columns

When a column has been in use for some time, it may be necessary to remove precipitated proteins or other contaminants which have built up on the gel bed.

- \* Only fresh buffer solutions should be used because many buffer substances are excellent supporters of microbial growth.
- \* The cleaning solutions should also be filtered before use.

Cleaning Sephadex G-types with 2 column volumes of a non-ionic detergent solution (1%). Sephadex may also be washed with 0.2 M NaOH on a Buchner funnel.

Cleaning Sepharose with a non-ionic detergent (1%).

Cleaning Sepharose CL with one bed volume of 0.5 M NaOH or a non-ionic detergent solution (1%)

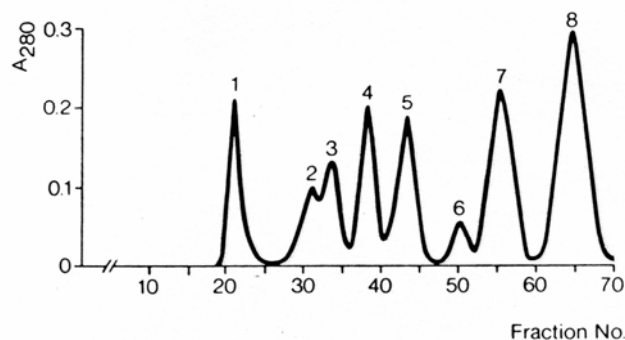
Cleaning Sephacryl HR with one or two column volumes of NaOH (0.2-0.5 M) at a flow rate which gives a contact time of at least 1 hour. The contact time is sufficient

to solubilize most protein precipitates. Sephacryl HR may also be washed with a solution of a non-ionic detergent.

- \* After cleaning, the column must be carefully re-equilibrated with 2-3 column volumes of eluent buffer before it is used again.

When the gel is to be repacked, follow the following procedures:

- 1) Put a large enough beaker under the column to collect the gel, remove the bottom piece and empty the column by pumping high purity water or buffer through it. Clean all column parts with soapy water or laboratory detergents. Inspect the top and bottom filters and change them.
- 2) Wash the gel with NaOH (0.1-0.2 M), then water and lastly 20% ethanol.
- 3) Resuspend the gel in at least 5 times the gel volume of high purity water in a beaker.
- 4) Allow the gel to sediment and pour off the supernatant.
- 5) Repeat the washing procedure once more before repacking the column.



**Fig. 8.** Gel filtration of proteins on Sepharose CL-6B under denaturing conditions.

Peaks, 1. Blue Dextran; 2. bovine serum albumin; 3. rabbit IgG H chain; 4.  $\alpha$ -chymotrypsinogen; 5. cytochrome c; 6. insulin; 7. B chain of insulin; 8. DNP-ala: Column, 1.5x90 cm: Eluent, 6 M guanidine-HCl, 0.1 M sodium phosphate, pH 8.0; flow rate, 6.8 ml.cm<sup>-2</sup>.h<sup>-1</sup>. (Ansari, A.A., Mage, R.G., J. Chromatogr. 140 (1977) 98-102. Reproduced by kind permission of the Authors and the Publisher).

## 5. Storage of gels and columns

- \* Any antimicrobial agent which does not interact with the gel may be used. For instance, 20% ethanol, 0.1-0.5 M NaOH, 0.05% sodium azide and 0.002% chlorhexidine.
- \* Used media should be stored at 4-8 °C, pH 6-8 in the presence of a suitable

bacteriostatic, e.g. 0.05% sodium azide or 20% ethanol.

## **V. Applications**

1. Fractionation by size:
2. Separation of monomers from dimers and higher aggregates:
3. MW estimation, native and other forms
4. Determination of equilibrium constants: Gel filtration has proved to be a valuable technique for the study of chemical equilibria. In the case of slow reactions, where the reactants and the products can be separated on a gel filtration column, these substances can be quantitatively determined in the effluent, thereby establishing the position of the equilibrium. Gel filtration can also be used to determine the position of equilibrium of complex formation where the reactions are rapid. In this case, one of the reactants is chromatographed in an eluent containing the other reactant.
5. Desalting: Gel filtration is particularly efficient for many everyday laboratory operations including
  - 1) buffer exchange
  - 2) phenol removal from preparations of nucleic acids
  - 3) removal of incorporated nucleotides during DNA sequencing
  - 4) removal of free low molecular weight labels, e.g.  $^{125}\text{I}$ ,  $^{32}\text{P}$ , FITC, from solutions of labelled proteins,
  - 5) removal of products, cofactors, inhibitors etc. from enzymes
6. Industrial applications

## Part 2: Ion Exchange Chromatography

\* An ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter-ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix.

ANION exchanger with exchangeable counter ions	CATION exchanger with exchangeable counter ions
Anion exchangers	Functional group
Aminoethyl (AE-)	$-\text{OCH}_2\text{CH}_2\text{NH}_3^+$
Diethylaminoethyl (DEAE-)	$-\text{OCH}_2\text{CH}_2\text{N}^+(\text{CH}_2\text{CH}_3)_2$
Quaternary aminoethyl (QAE-)	$-\text{OCH}_2\text{CH}_2\text{N}^+(\text{C}_2\text{H}_5)_2\text{CH}_2\text{CH}(\text{OH})\text{CH}_3$
Cation exchangers	
Carboxymethyl (CM-)	$-\text{OCH}_2\text{COO}^-$
Phospho	$-\text{PO}_3\text{H}_2^-$
Sulphopropyl (SP-)	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-$

- \* The presence of charged groups is a fundamental property of an ion exchanger. The type of group determines the type and strength of the ion exchanger; their total number and availability determines the capacity.
- \* The separation in ion exchange chromatography is obtained by reversible adsorption. Most ion exchange experiments are performed in two main stages. The first stage is sample application and adsorption. Un bound substances can be washed out from the exchanger bed using a column volume of starting buffer. In the second stage, substances are eluted from the column, separated from each other. **The separation is obtained since different substances have different affinities for the ion exchanger due to differences in their charge.** These affinities can be controlled by varying conditions such as ionic strength and pH.

- \* Ion exchange chromatography is capable of separating species with very minor differences in properties, such as two proteins differing by only one amino acid. Therefore, it is a very powerful separation technique.

### **Sephadex ion exchangers**

- \* Sephadex ion exchangers are produced by introducing functional groups onto Sephadex, a cross-linked dextran. These groups are attached to glucose units in the matrix by stable ether linkages.
- \* Sephadex ion exchangers are stable in all solvents. They are stable in water, salt solutions, organic solvents, alkaline and weakly acidic solutions. They can also be used in the presence of denaturing solvents.
- \* Exposure to strong oxidising agents or dextranases should be avoided. During regeneration, the ion exchanger can be exposed for a short time to 0.01 M NaOH or 0.2 M HCl without appreciable hydrolysis.
- \* Sephadex ion exchangers can be sterilized by autoclaving for up to 30 min at 120 °C, at neutral pH in the salt form.
- \* **Swelling:** Due to the presence of charged groups in the matrix, the swelling varies with ionic strength and pH. The degree of swelling decreases with increasing ionic strength or at pH values close to the pK of the charged groups.
- \* **Flow rates:** Flow rates can be expressed either as ml.h<sup>-1</sup> or as cm.h<sup>-1</sup>. Flow rate of Sephadex column is dependent on the bed height. During experiments which use ionic strength gradients, the bed height decreases with increasing ionic strength, due to Sephadex shrinking, and this results in a steady increase in flow rate. It is therefore necessary **to use a pump to maintain a steady flow rate** throughout the experiment using Sephadex column.

### **Sepharose ion exchangers**

- \* Sepharose ion exchangers are prepared by cross-linking agarose with 2,3-dibromopropanol and desulphating the resulting gel by alkaline hydrolysis under reducing conditions.
- \* The Sepharose CL-6B matrix has much greater rigidity for equivalent porosity than a Sephadex gel would have.
- \* Sepharose ion exchangers are stable in all solvents. They are stable in water, salt solutions, organic solvents in the range pH 3-10. Prolonged exposure of DEAE-Sepharose CL-6B to very alkaline conditions should be avoided because of the

inherent instability of the DEAE-group as a free base.

- \* Both DEAE- and CM-Sepharose CL-6B can be used in solutions of non-ionic detergents such as Triton X-100. They can also be used with strongly dissociating solvents such as 7M urea.
- \* Sepharose ion exchangers can be used at temperatures up to 70 °C and can be sterilized by autoclaving for up to 30 min at 120 °C, at neutral pH in the salt form.
- \* Swelling: The cross-linked nature of the matrix means that the bed volume changes very little with changes in ionic strength or pH.
- \* Flow rates: The cross-linked nature of Sepharose ion exchangers means that the very high flow rates can be obtained.

## DEAE-Sepharcel

- \* DEAE-Sepharcel is a bead-formed cellulose ion exchanger produced from high purity cellulose.
- \* DEAE-Sepharcel is stable in aqueous suspension within the range pH 2-12. Strong oxidizing agents should be avoided.
- \* The cross-linked bead form of DEAE-Sepharcel has a stable bed volume over a wide range of ionic strengths and pH values.
- \* Flow rates: The rigidity of DEAE-Sepharcel enables high flow rates to be used. Flow rates of 10 cm.h<sup>-1</sup> are usually suitable for the resolution of protein mixtures.

## Ion Exchanger Preparation

### 1. Choice of ion exchanger matrix

- \* If the sample components are most stable below their pI's, a cation exchanger should be used. If they are most stable above their pI's, an anion exchanger should be used. If stability is high over a wide pH range, either type of ion exchanger can be used.

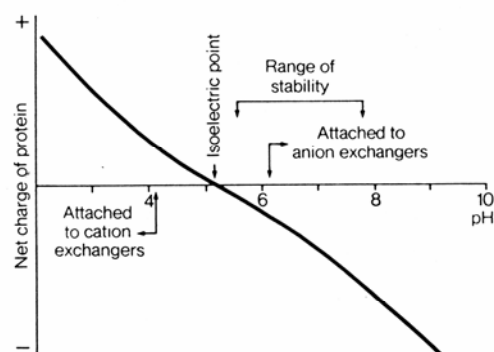


Fig. 17. The net charge of a protein as a function of pH. The pH ranges in which the protein is bound to anion or cation exchangers and an arbitrary range of stability are shown.

- \* Ion exchangers should be swollen at the pH to be used in the experiment. Swelling at high temperature (2 h in a boiling water bath) also serve to deaerate the gel. Vigorous stirring with a magnetic stirrer should be avoided in order not to damage the beads. Remove the supernatant and replace with fresh buffer several times during the swelling period.
- \* If ion exchangers are to be used with counter-ions other than those supplied (i.e. other than sodium or chloride) then the following procedure should be used: suspend the required amount of ion exchanger in an excess of 0.5-1.0 M solution of the salt of the new counter-ion. After sedimentation and decantation resuspend the ion exchanger in the buffer to be used in the experiment. Repeat several times.
- \* For the best resolution in chromatography, it is not advisable to use more than 10-20% of the capacity. This value can be exceeded if resolution is adequate. The available capacity of ion exchanger can be determined by a test-tube experiment. From the experiment, the maximum volume or weight of sample that can be applied to a given volume of ion exchanger can be determined. However, this figure should be multiplied by a factor of 5 to 10 to obtain the amount of ion exchanger required for chromatography.

## 2. Choice of buffer pH and ionic strength

- \* The starting pH should be about 1 pH unit above the  $p_i$  of the substances of interest with anion exchangers and 1 pH unit below the  $p_i$  with cation exchangers. The pH to be used in the experiment should allow the substance to be bound, but should be as close to the point of release as possible. Therefore, starting conditions should be sufficiently close to eluting conditions so that elution is neither time consuming nor involves harsh conditions.

**Table 7.** Buffers for ion exchange chromatography.

Ion exchanger	Recommended buffer ions	Starting pH
Anion exchanger	Cationic e.g. alkylamines, aminoethyl alcohol, ammonium, ethylenediamine, imidazole, Tris, pyridine etc.	1 pH unit above $p_i$ of protein or as determined empirically.
Cation exchanger	Anionic e.g. acetate, barbiturate, citrate, glycine, phosphate etc.	1 pH unit below $p_i$ of protein or as determined empirically



\* Test-tube method for selecting starting pH (if the  $p_i$  of the sample is unknown)

- 1) Set up a series of 10 x 15 ml test-tubes.
- 2) Add 0.1 g Sephadex ion exchanger or 1.5 ml Sepharose or Sephacel ion exchanger to each tube.
- 3) Equilibrate the gel in each tube to a different pH by washing 10 times with 10 ml of 0.5 M buffer. Use a range of pH 5-9 for anion and pH 4-8 for cation exchangers with 0.5 pH unit intervals between tubes.
- 4) Equilibrate the gel in each tube at a lower ionic strength (0.05 M for Sephadex or 0.01 M for Sepharose and Sephacel ion exchangers) by washing 5 times with 10 ml of buffer of the same pH but lower ionic strength.
- 5) Add a known constant amount of sample to each tube.
- 6) Mix the gel for 5-10 minutes
- 7) Allow the gel to settle.
- 8) Assay the supernatant for the substance.

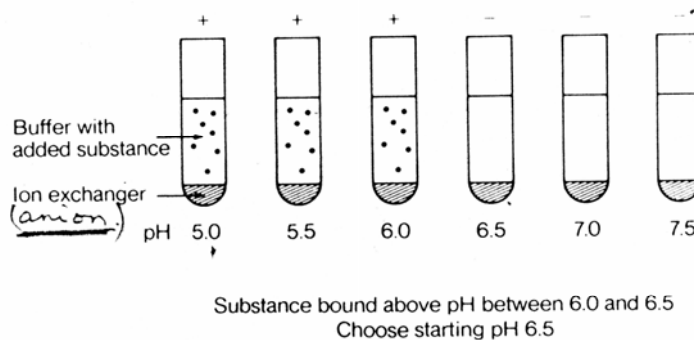


Fig. 18. Test-tube method for selecting ion exchange conditions.

- \* The highest ionic strength which permits binding of the substances of interest and the lowest ionic strength that effects elution should normally be used as the starting and eluting ionic strengths.
- \* Salts also play a role in stabilizing protein structures in solution and so it is important that ionic should not be too low in order to prevent protein denaturation.
- \* The variation in swelling of Sephadex ion exchangers is less marked at high ionic strengths than at low ionic strengths. Problems of swelling and contraction are thus minimized by choosing the highest possible ionic strength. A simple test can be performed to determine binding and eluting ionic strengths.
- \* Test-tube method for selecting starting ionic strengths
  - 1) Set up a series of tubes with ion exchanger as described above

- 2) Equilibrate the gel in each tube with 0.5 M buffer at the selected stalling pH (10 x 10 ml washes).
- 3) Equilibrate the gel in each tube to a different ionic strength using a range from 0.05 M to 0.5 M NaCl for Sephadex ion exchangers and from 0.01 M to 0.3 M NaCl for Sephacel and Sepharose ion exchangers at constant pH.  
This will require 5 x 10 ml washes. Intervals of 0.05 M NaCl are sufficient.
- 4) Add sample and assay supernatant as described previously.

### **3. Choice of column**

#### **1) Column height and diameter:**

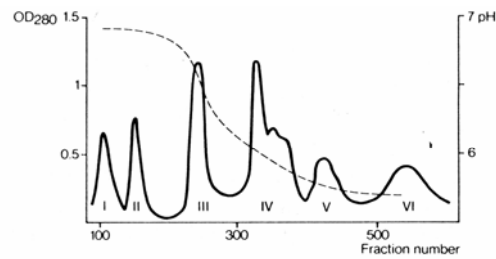
- \* Conditions should be chosen such that the sample substances are adsorbed within the upper 1-2 cm of the ion exchanger bed. A bed height of 20 cm is often sufficient. Longer beds may be necessary for resolution of very complex mixtures.
- \* If the bed height is fixed, the column diameter can be calculated on the basis of how much ion exchanger is to be packed in the column. Column diameter is, therefore, dependent on the required capacity of the ion exchanger bed.

#### **2) Packing the column:**

- \* Packing an ion exchange column is usually easier than packing a gel filtration column since the bed height required is usually much less.
- \* Allow the ion exchanger to pack under constant pressure. A slightly higher flow rate should be used for packing than is to be used in the experiment.
- \* Run at least two bed volumes of buffer through the ion exchange bed in order to allow the system to reach equilibration and to stabilize the bed.
- \* The bed should be checked for irregularities or air bubbles using transmitted light 4. from a lamp held behind the column.

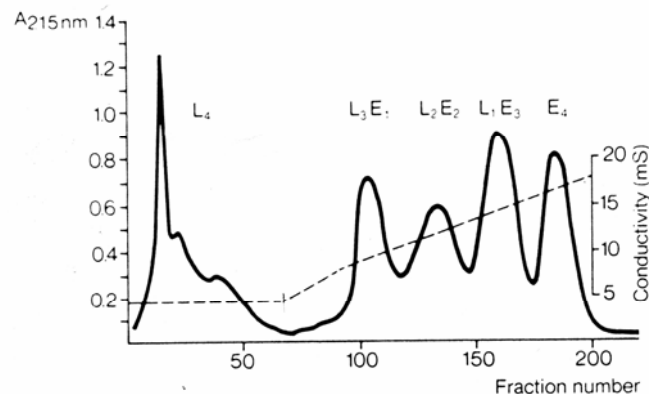
### **Elution**

- \* When conditions have been chosen so that the substances of interest are bound to the gel, elution can be achieved by varying either buffer pH, ionic strength, or possibly both.
- \* Change of pH: Altering the pH towards the isoelectric point of a substance causes it to lose its net charge, desorb, and elute from the ion exchanger.



**Fig. 23.** Elution pattern of whole stripped haemocyanin on DEAE-Sepharose CL-6B. Sample applied in 0.1 M sodium phosphate buffer pH 6.8 and eluted by a descending pH gradient. (Lamy, J., Lamy, J., Weill, J. *Arch. Biochem. Biophys.* 193 (1979) 140—149. Reproduced by kind permission of the authors and publisher).

\* **Change of ionic strength:** At low ionic strengths, competition for chained groups on the ion exchanger is at a minimum and substances are bound strongly. Increasing the ionic strength increases competition and reduces the interaction between the ion exchanger and the sample substances, resulting in their elution. Note that it is important to wash the column with starting buffer before starting the gradient.



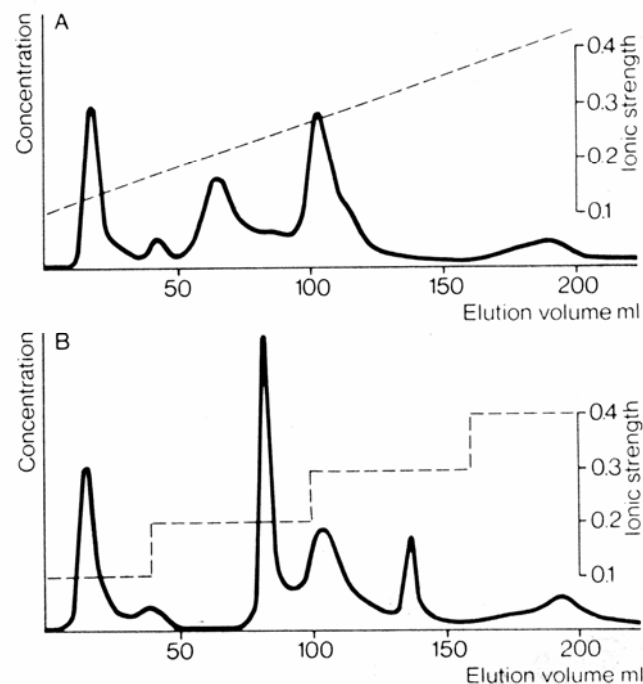
**Fig. 24.** Elution pattern of mixtures of phytohaemagglutinin isolectins on SP-Sephadex. Column:  $1.5 \times 45$  cm. Eluent: 0.05 M potassium phosphate, pH 6.0, with a linear salt gradient to 0.188 M NaCl. Fraction size: 5 ml. L = lymphocyte reactive subunit. E = erythrocyte reactive subunit (Felsted, R.L., Egorin, M.J., Leavitt, R.D. et al. *J. Biol. Chem.* 252 (1977) 2967—2971. Reproduced by kind permission of the authors and publisher).

\* **Ionic strength gradients** are easily produced by filling each chamber with the same buffer at different ionic strengths. The mixing chamber should contain the starting buffer and the other chamber the limiting buffer.

\* **Continuous ionic strength gradients** are easy to prepare and very reproducible. Two buffers of differing ionic strength are mixed together and if the volume ratio is

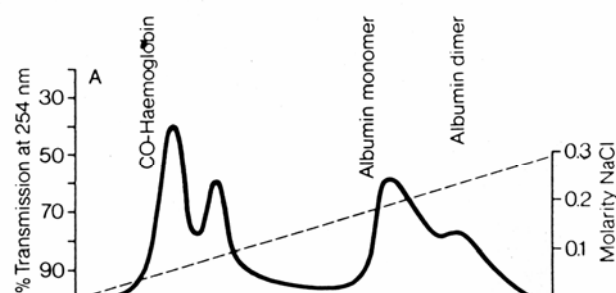
changed linearly, the ionic strength changes linearly. Linear gradients help substances to be eluted in symmetrical peaks and give better resolution.

\***Stepwise** ionic strength gradients are produced by the sequential use of the same buffer at different ionic strengths.



**Fig. 25.** Continuous and stepwise gradient elution of bovine serum on QAE-Sephadex A-50. Bed dimensions:  $1.5 \times 26$  cm (column K 15/30). Sample: 4 ml 3% (w/v) lyophilized bovine serum. Eluent: 0.1 M Tris-HCl buffer, pH 6.5. Curve A was obtained using a continuous NaCl gradient to 0.5 M NaCl. Curve B was obtained using a stepwise NaCl gradient. Flow rate: 0.2 ml/min. The first peak in both diagrams represents IgG. The fourth peak in curve A represents serum albumin. Stepwise elution caused the albumin to be eluted in two peaks (4 and 5). Other peaks were not identified. (From Pharmacia Fine Chemicals, Uppsala, Sweden).

\* The total volume of eluent in a gradient should be about five times the bed volume of the ion exchanger. Longer gradients may lead to excessive band spreading and dilution, whilst short (steep) gradients release substances close together and may not give adequate separation.



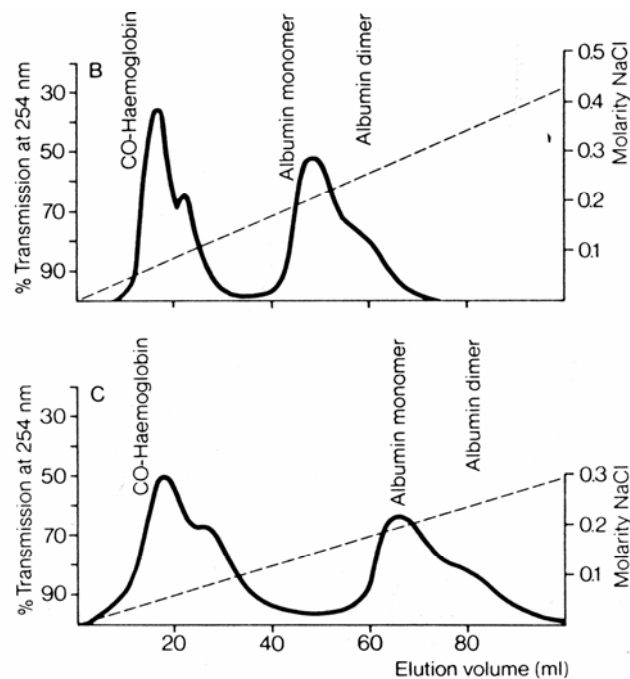


Fig. 33. Separation of haemoglobin and albumin on DEAE-Sephadex A-50. Eluent: 0.1 M Tris-HCl buffer pH 8.3. The sample consisted of a mixture of O<sub>2</sub>-haemoglobin, CO-haemoglobin, albumin monomer and albumin dimer. In cases A and B the flow rate was the same, but the gradients differed in steepness. Cases A and C differed in flow rate. In A the rate of flow was 8 ml. h<sup>-1</sup> and in C, 20 ml. h<sup>-1</sup>. (From Pharmacia Fine Chemicals AB, Uppsala, Sweden).

## Regeneration

- \* Washing with a high salt solution is adequate to remove most substances after a run.

## Storage

- \* All swollen ion exchangers should be stored in their salt form, in buffered suspension containing an antimicrobial agent.
- \* Antimicrobial agents for anion exchangers: Phenyl mercuric salts, 0.001, effective in weakly alkaline solutions. Hibitane (chlorohexidine) 0.002.
- \* Antimicrobial agents for cation exchangers: Merthiolate 0.005, mostly effective in weakly acidic solution.
- \* Antimicrobial agents for anion and cation exchangers: Chlorexone (trichlorobutanol), 0.05%, effective only in weakly acidic solutions.