METHODOLOGICAL ASPECTS OF GEL FILTRATION WITH SPECIAL REFERENCE TO DESALTING OPERATIONS

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The ability of porous bodies to distinguish between molecules of different sizes has been repeatedly observed. The regular structure of zeolites results in an abrupt change in adsorptive properties at a definite molecular size[1]. This has been utilized to obtain separations among gases and organic substances with a small number of atoms. The porous structure of the synthetic ion-exchange resins is due to the swollen three-dimensional network in which the size of the meshes determines the porosity. According to the nature of the synthetic procedure they vary in size and their structure is much less defined than in the crystalline zeolites. Nevertheless they are quite useful for separations according to size as has been shown by WHEATON AND BAUMAN[2]. The molecular sieve effect has been utilized to increase the selectivity in ion-exchange operations, *e.g.* in the separation of cellulose xanthate from small ions[3], to separate polygalacturonic acid from galacturonic acid[4] and to fractionate peptides of different sizes[5].

Networks of polar character and devoid of charged groups do not exchange or exclude ions and thus solutes behave in a similar manner no matter if they are charged or not. In columns packed with swollen starch LINDQVIST AND STORGÅRDS[6] separated peptides of different sizes and LATHE AND RUTHVEN[7] demonstrated that for a large number of solutes the elution volume varied with the size of the solute.

The use of particular dextran gels for separations according to size was introduced by PORATH AND FLODIN[8]. The method was named gel filtration as suggested by TISELIUS. A study of the conditions for separation of amino acids, peptides and proteins has been published by PORATH[9]. Two gels with different degrees of cross-linkage were compared. This was also done by FLODIN AND GRANATH[10] with fractions of dextran as solutes. As expected the range of separation increased with decreasing degree of cross-linkage. A fractionation of enzymes from snake venoms has been made with the aid of highly swelling gels[11]. The purification of pepsin on a preparative scale[12] and the fractionation of extracts from pituitary posterior lobes[13] have been described. By using dextran gels of small particle size it was possible to separate molecular species that differed only slightly in size, as was shown for cellodextrins, where the oligosaccharides up to cellohexaose appeared as well-separated components[14]. Effects due to adsorption onto the gel matrix have sometimes been observed. A thorough study of a large number of solutes[15] revealed certain regularities.

With distilled water as eluant the behaviour of many solutes was irregular, but as soon as salt was present some of these disappeared. Some solutes of aromatic character displayed adsorption effects independent of the presence of salt.

The purpose of this paper is to study some factors influencing the removal of salts from colloids, notably proteins, by gel filtration. The conditions are chosen so as to give a hint as to the optimal conditions for transferring a protein to a new salt medium. This type of operation must often be used prior to electrophoresis or ion-exchange chromatography.

EXPERIMENTAL

Materials

The dextran gel used was Sephadex G-25 (Pharmacia, Uppsala, Sweden) lots number 125 and 161. Their water regain was 2.3 g of water per g of dry material. The particle size was 50–270 mesh on the U.S. Standard screen series, as measured by analysis of the dry material. In most experiments fractions with a narrower size distribution were used. They were obtained by dry screening.

The haemoglobin was prepared by haemolysis of washed bovine erythrocytes and lyophilization. It contained an appreciable amount of methaemoglobin. Carbon-monoxy-haemoglobin was prepared from bovine erythrocytes according to the method of PRINS[16]. All other chemicals were *pro analysi* or of a comparable grade of purity.

Methods

Preparation of gels. The dry Sephadex powder was suspended in tap water and stirred for a few minutes to allow it to swell. After a sedimentation time of ½ to 1 hour the fines remaining in the supernatant were removed by decantation. The procedure was repeated at least five times. The volume of water was chosen so that the ratio supernatant to sediment was at least 10:1.

Packing. The columns used were cylindrical glass tubes of 2 and 4 cm diameter and 40 to 100 cm in length. At the bottom they were joined to a 5 cm long capillary with a 1 mm bore.

The procedure used was essentially that described by FLODIN AND KUPKE[17] for cellulose columns.

Before packing, the column was mounted vertically and filled with tap water at room temperature. A small piece of glass wool was laid over the outlet capillary, and above it a 2 cm layer of glass beads (diameter 0.5 mm). The top of the column tube was connected to a 1 l funnel through a 100 cm long glass tube with half the column diameter and inserted through a rubber stopper. The arrangement is shown in Fig. 1. The system was then filled with water up to the funnel. Care was taken that no air bubbles were present in the columns.

The water-swollen Sephadex was then added to the funnel and agitated with a motor-driven stirrer during the packing procedure. The bottom outlet from the column was opened to allow a flow rate of 5 to 40 ml per minute. When the suspended particles reached the bottom of the column the flow was stopped for a long enough time for a 1 to 2 cm high bed to be formed, whereupon the flow was started again.

A rising horizontal boundary of packed material was considered evidence of good packing. To compensate for the increasing resistance to flow with proceeding packing the outlet tube was progressively opened wider. After all the material had been packed, the funnel and connecting tube were removed and a filter paper with a

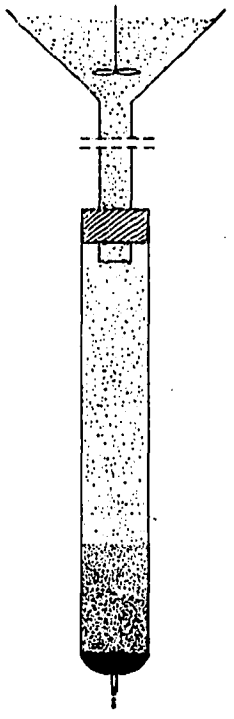

Fig. 1. Arrangement for packing of the columns.

diameter slightly smaller than the bore of the column tube was placed on the horizontal even surface of the bed. To let the bed stabilize it was percolated for at least a couple of hours, preferably overnight, with the eluant to be used.

Application of the sample. Most of the liquid over the bed was removed and the last few ml were allowed to pass into it. At the moment when the surface was about to dry out the sample was carefully added dropwise from a pipette. Then the flow was started and the sample allowed to enter the bed. At the moment it disappeared a few ml of the eluant were added to wash the surface. Finally, the space above the bed was filled with eluant.

Elution. The top end of the column tube was closed by a rubber stopper through which the eluant was fed. When low viscosity samples were run, a constant flow rate was obtained by means of a Mariotte flask. A constant-feeding pump (Sigma-motor) was used when the viscosity of the samples was high or when a more precise control of flow rate was necessary. The eluates were collected in a fraction collector. In most experiments a time-regulated collector (Stålprodukter, Uppsala, Sweden) was used. In the study of the influence of particle size and flow rate a volume-regulated collector was used (Radi Rac, LKB-Produkter, Stockholm, Sweden). All experiments were made at room temperature.

Control of packing. The homogeneity of packing was checked by passing a zone of haemoglobin through the bed. The column was repacked if the zone became skew during the passage. All experiments with haemoglobin were made with an eluant containing salt to avoid adsorption of denatured protein. In the cases where no electrolytes were present in the eluant the column was checked by passing a zone of dilute india ink as described by LATHE AND RUTHVEN[7].

To determine the void volume the elution curve for a narrow zone of haemoglobin was taken. Since haemoglobin is completely excluded from the gel particles the position of the maximum of the curve represented the void volume. A quantitative check of the column performance was sometimes made by eluting a sample the volume of which was 10% of the bed volume. It was considered satisfactory when dilution during the passage was less than twofold.

Preservation of the beds. To avoid microbial growth the buffers were stored over chloroform.

Analytical methods. The ultraviolet light absorbing substances were analysed in a Unicam SP 500 spectrophotometer. The chloride ion determinations were made by the Volhard titration procedure. The conductivity measurements were made with a Conductolyzer (LKB-Produkter, Stockholm, Sweden).

RESULTS

Flow rate and particle size

To investigate the influence of flow rate and particle size on the efficiency of a column, experiments were made with uridylic and hydrochloric acids. They are retained to the same degree by the column, but differ in molecular weight and thus their diffusion coefficients are different. In order to make the experiments strictly comparable they were performed in columns with as nearly equal dimensions as possible and with narrow sieve fractions from the same batch of dextran gel. The dimensions of the columns and the sieve fractions are given in Table I. The volume of the sample was 3 ml throughout and the concentrations were 0.358 and 237 mg per ml for uridylic acid and hydrochloric acid, respectively. Fractions of 2 ml were taken and analysed by U.V.-absorption at 260 mμ and by titration with sodium hydroxide, respectively.

TABLE I

COLUMN DIMENSIONS

Column No.	*Sieve fraction mesh*	*Test substance*	*Column dimensions cm × cm*
1.	50– 80	uridylic acid	2 × 65
2.	140–200	uridylic acid	2 × 65
3.	270–400	uridylic acid	2 × 67
4.	50– 80	hydrochloric acid	2 × 67
5.	270–400	hydrochloric acid	2 × 70.5

From the elution curves the number of theoretical plates was calculated according to GLUECKAUF[18]. For curves having the shape of gaussian error curves the plate number is

$$N = 8\left(\frac{V_{max}}{\varepsilon}\right)^2$$

where ε is the band width at the height c_{max}/e and V_{max} is the elution volume for the maximum concentration (c_{max}). The equivalent height per theoretical plate (EHTP) was obtained by dividing the height of the column by the number of theoretical plates. In Tables II and III the values obtained under varying experimental conditions are given.

TABLE II

EHTP VALUES IN mm FOR URIDYLIC ACID

v ml/h	*Sieve fraction, mesh*		
	50–80 (column 1)	*140–200 (column 2)*	*270–400 (column 3)*
10	0.39	0.102	0.15
24	0.72	0.182	0.11
51	1.49	0.21	
90	2.62		
130	4.14		
190	5.49		

In Fig. 2A are shown three of the elution curves obtained with uridylic acid in column 1 when the flow rate was varied. At a rate of 24 ml per hour in columns 1 to 3 the curves given in Fig. 2B were obtained.

For uridylic acid the EHTP in column 1 was proportional to the rate of flow. With hydrochloric acid the increase with flow rate was small and the efficiency was practically constant within the range investigated. Higher values for the EHTP were obtained at the lowest flow rates indicating longitudinal diffusion or convection. Decreasing particle size decreased the EHTP for both solutes. The values in Tables II

TABLE III

EHTP VALUES IN mm FOR HYDROCHLORIC ACID

v ml/h	*Sieve fraction, mesh*	
	50–80 (column 4)	*270–400 (column 5)*
14	1.05	0.23
24	0.69	0.26
48	0.44	
70	0.45	
96	0.51	

and III show that the most efficient way to increase the performance of a column is to decrease the particle size of the dextran gel.

A few experiments with smaller sample volumes were made in column 3 in order to measure the accuracy of the EHTP. However, the observed changes in the EHTP were small.

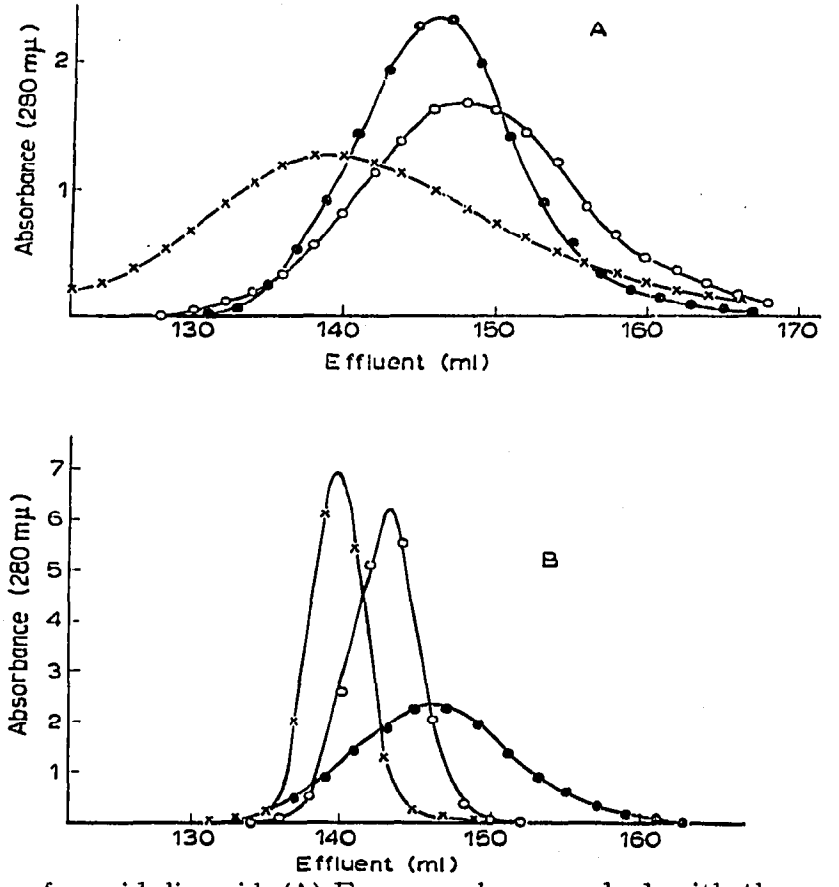

Fig. 2. Elution curves for uridylic acid. (A) From a column packed with the 50–80 mesh fraction and the elution rates 24 ml (●—●—●), 90 ml (o—o—o), and 190 ml (×—×—×) per h. (B) At a constant elution rate of 24 ml/h from columns packed with the 50–80 mesh (●—●—●), 140–200 mesh (o—o—o), and 270–400 mesh (×—×—×) sieve fraction.

Viscosity

In a column with an original height of 85 cm and 4 cm in diameter a series of experiments was made in order to evaluate the influence of the viscosity of the sample on the efficiency of the column. The 100 ml samples contained 0.1 g haemoglobin and 1 g of sodium chloride and varying amounts of a dextran fraction with limiting viscosity number (η) equal to 0.68 (M_w = 1,800,000). In Table IV the compositions of the solutions and their viscosities are shown. The eluant was 0.1 *M* phosphate buffer of pH 7.0. The bed was eluted at a rate of 180 ml per hour. The elution curves for haemoglobin and sodium chloride were measured and can be seen in Fig. 3A, B and C. The dextran distribution is closely similar to that of the haemoglobin.

In experiment 1 the peaks were very unsymmetrical and there was considerable overlapping. With lower sample viscosity the peaks became more symmetrical

TABLE IV

DEXTRAN CONCENTRATION AND VISCOSITY OF SAMPLES

Experiment No.	*Dextran %*	*$\eta_{rel.}$ centipoise*
1.	5	11.8
2.	2.5	4.2
3.	1	2.0
4.	0	1.0

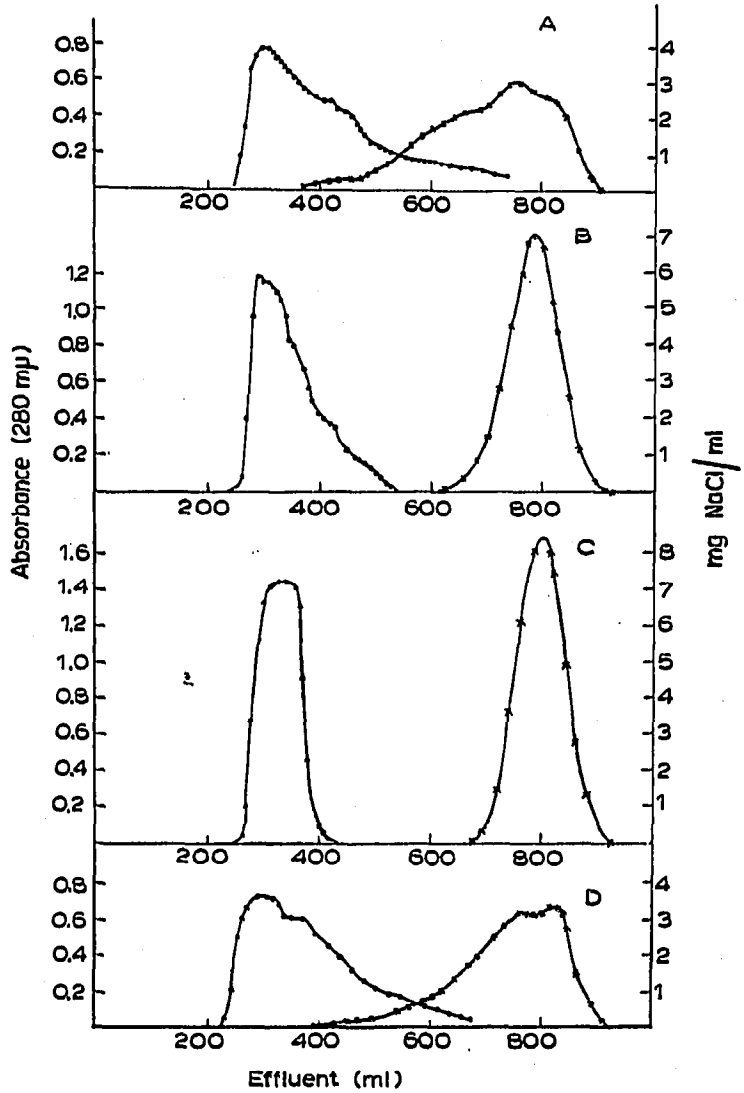

Fig. 3. Influence of viscosity. Elution curves for haemoglobin (o—o—o) and sodium chloride (×—×—×) in the presence of varying amounts of dextran. At an elution rate of 180 ml/h with (A) 5% dextran, (B) 2.5% dextran, and (C) without dextran. (D) 5% dextran and an elution rate of 36 ml/h.

(Fig. 3B) and in the absence of dextran (Fig. 3C) they were almost perfectly symmetrical. An interesting feature is that the fast component has a sharp front and a diffuse rear, while the opposite is true for the slow one. A partial explanation for this is that the bed was compressed while the viscous samples were in the column.

An experiment was made with the most viscous solution ($\eta_{rel.}$ = 11.8 cp) and a flow rate of only 31 ml per hour. From Fig. 3D it is evident that the resolution was somewhat improved, although the difference was small. Reducing the viscosity was much more efficient.

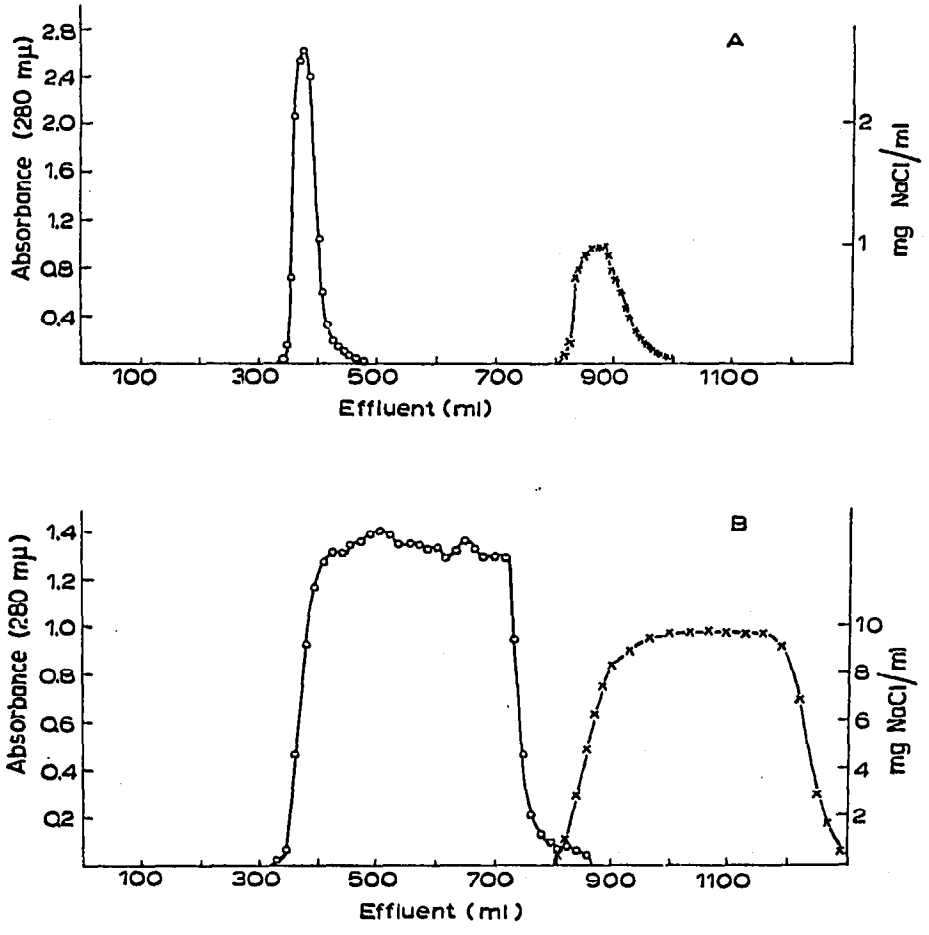

Fig. 4. Elution curves for haemoglobin (o—o—o) and sodium chloride (×—×—×) at an elution rate of 240 ml/h. (A) Sample volume 10 ml. (B) Sample volume 400 ml.

Sample volume

In Figs. 4A and B the elution curves for two experiments are shown, in which the volume of the sample differed by a factor of forty. They were performed in a 4 cm × 85 cm bed (volume = 1070 ml) packed with 100–200 mesh dextran gel. In the first experiment 10 ml of a solution containing haemoglobin and sodium chloride (100 mg of each) were applied and in the second one a 400 ml sample containing 400 mg haemoglobin and 4 g sodium chloride. In both experiments the haemoglobin appeared in the effluent at the same breakthrough volume. A complete separation

was not obtained in the second experiment although about 99 % of the haemoglobin was free from salt. It is of interest to compare the dilution of the protein in the experiments. In the first one it was diluted 10 times and in the second 1.25 times. In the second experiment the salt was diluted 1.35 times.

Buffer concentration

The influence of the amount of buffering salts present in the eluant was studied with samples of the same concentration of CO-haemoglobin. The sample volume was kept to approximately 10 % of the bed volume. The experimental conditions are given in Table V. The columns were packed with the 100–200 mesh sieve fraction.

TABLE V

EXPERIMENTAL CONDITIONS

Experiment No.	*Column dimensions cm × cm*	*Eluant*	*Sample volume ml*
1.	4 × 36	0.1 *M* phosphate, pH 5.9	50
2.	4 × 36	0.05 *M* phosphate, pH 6.0	50
3.	4 × 75	0.01 *M* phosphate, pH 6.2	100

The samples were prepared by diluting a 10 % stock solution of CO-haemoglobin containing sodium chloride with an equal volume of the eluant in experiments 1 and 3 and with an equal volume of water in experiment 2. The elution rate was 120 ml per hour and the effluent collected in 15 ml fractions. The protein and chloride ion concentrations were measured in each fraction and the pH and conductivity in every second one. Experiments 1 and 2 gave similar results and in Fig. 5 experiment 2 is illustrated.

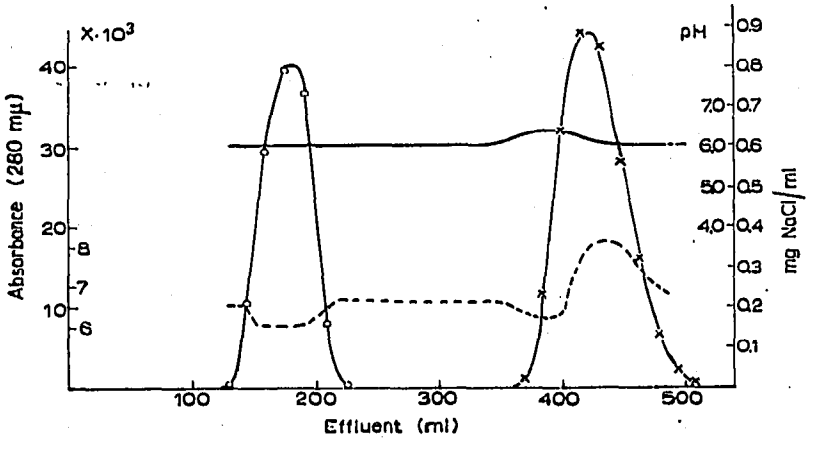

Fig. 5. Elution curves for CO-haemoglobin (o—o—o) and sodium chloride (×—×—×) at an elution rate of 120 ml/h. Eluant: 0.05 *M* sodium phosphate buffer. The solid horizontal line represents the pH and the broken line the conductivity.

It is seen that the buffer was of sufficiently high concentration to shift the pH to that of the buffer. A zone of higher pH appears immediately before the chloride zone and corresponds to the amount of hydrogen ions consumed. The minimum in

conductivity in the protein peak shows that a Donnan equilibrium has been established. In Fig. 6, illustrating experiment 3, the zone of higher pH is much broader and has just separated from the protein peak. The excess of electrolytes was removed as usual but the titration of the protein was evidently not finished until it had travelled down the greater part of the column. The conductivity curve was correspondingly more irregular in this experiment.

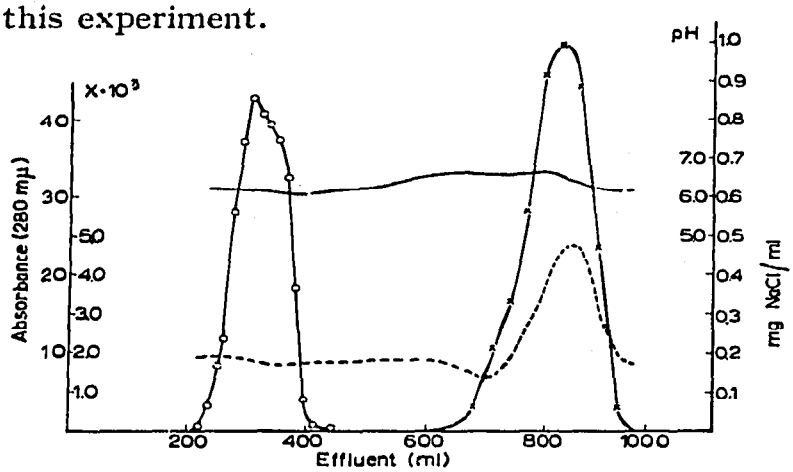

Fig. 6. Elution curves for CO-haemoglobin (o—o—o) and sodium chloride (×—×—×) at an elution rate of 120 ml/h. Eluant: 0.01 *M* sodium phosphate buffer. The horizontal solid line represents the pH and the broken line the conductivity.

DISCUSSION

As is the case in liquid–liquid partition chromatography, in gel filtration the solutes are distributed between a mobile and a stationary phase. However, the composition of the phases is the same in the latter method and the stabilizing substance is not passive as it is supposed to be in partition chromatography, but has a decisive influence on the process. The function of the swollen dextran gel is not only to stabilize the stationary phase, but also to provide a defined three-dimensional network having the property to sort molecules according to their size.

In analogy with the liquid–liquid partition chromatographic systems, it is possible to define a parameter for a solute, the distribution coefficient (K_D), which is independent of the geometry of the column. In the following the calculation and significance of this coefficient is discussed.

The total volume of the gel bed is

$$V_t = V_0 + V_i + V_g$$

where V_0 is the void volume, V_i the volume of the stationary phase and V_g the volume of the gel matrix. The weight of the stationary phase is calculated from the known amount of dry gel material (a) in grams and its water regain (W_r), defined as the number of grams of water held by one gram of dry material. The latter quantity is constant over a wide range of pH and ionic strength in the presence of many common salts and buffer substances. Notable exceptions are solutions containing borate or hydroxyl ions. If the density of water is taken as unity

$$V_i = aW_r$$

As a consequence of this definition V_i includes the water of hydration, which presumably is inactive as solvent and tends to lower the values for the distribution coefficients.

The void volume is easily determined by passing through the column a zone of a solute known to be completely excluded from the gel grains.

Sometimes the volume of the stationary phase must be calculated indirectly, for instance, when some gel has been lost during the back-wash. It is given by the formula

$$V_i = \frac{dW_r}{W_r + 1}(V_t - V_0)$$

where d is the true wet density of the swollen gel particles.

The distribution coefficient is given through the relationship

$$K_D = \frac{V_e - V_0}{V_i}$$

where V_e is the elution volume of the solute.

In partition chromatography the distribution coefficient is defined as the ratio of the concentrations in the mobile and stationary phases. For a molecular sieve mechanism, such as the one described in the present paper, some parts of the stationary phase are available to the solute while others are not. The coefficients are thus a measure of the part of the phase that contains solute of the same concentration as the mobile phase.

A zero value for the distribution coefficient means complete exclusion from the gel particles. Values between zero and unity mean either partial penetration of the particles or that adsorption occurs, or both. Adsorption is indicated when the value is higher than unity.

The elution volume of a solute is

$$V_e = V_0 + K_D V_i$$

and two solutes with the distribution coefficients K_D' and K_D'' appear in the effluent separated by a volume equal to

$$V_i(K_D' - K_D'')$$

To obtain a complete separation the sample volume must be less than this value. How much less depends on a number of factors, among which the particle size, the flow rate and the viscosity of the sample are the most important ones.

The volume of the stationary phase is generally in the range of 40–60% of the total bed volume. It is therefore of great importance that the packing is made carefully. Even a moderately skewed zone reduces the separation efficiency considerably. The method of packing described in this paper gives good results, but is not entirely reliable and checking the column by passing a coloured zone is therefore recommended.

If the packing of the column and the application of the sample and the removal of the zones could be made perfectly, the shape of the column would be of minor importance. However, experience has shown that the best results are obtained when the ratio of height to diameter is large. With the type of equipment used in this investigation, a ratio of at least 10 to 1 gives excellent results. Satisfactory separations have been obtained with a 4 to 1 ratio, although disturbances due to uneven packing are less easily suppressed.

The variations in particle size and flow rate prove the former factor to be the most important one for the efficiency of the column. A large number of theoretical plates, and thus improved column efficiency, is best obtained by using a small particle size. However, a small particle size increases the pressure drop over the column and hence decreases the flow rate. A compromise between the time to be allowed for an experiment and the particle size is therefore necessary. For the type of separations described in this paper, the 100–200 mesh fraction gives excellent results. The experiment may be completed in less than one day, in many cases in one to two hours. Since a completely excluded solute remains in the column only about one third of the time for the total experiment, an experiment with a protein may often be made at room temperature without risk of denaturation. However, if desirable, the experiments can be conveniently made close to the freezing point.

The importance of the particle size and flow rate strongly indicates diffusion as the rate-determining factor in the gel filtration process. The fact that uridylic acid is much more sensitive to variations than hydrochloric acid is explained by their different diffusion rates. The spreading of solutes incapable of penetrating the gel phase is due to eddy formation, channelling and wall effects.

The gel filtration procedure is limited by the viscosity of the sample. A viscous zone spreads as a result of irregularities in the flow pattern. In the experiments described in this paper, varying amounts of dextran were added to the samples in order to vary this parameter. The haemoglobin and the dextran appeared simultaneously in the effluent and the spreading of the former substance was a consequence of the presence of dextran. The pressure drop over the bed increased and resulted in a compression of the bed. This was only manifested in a decrease of the void volume and not of the stationary phase. Thus it is not a true compression of the gel particles but a deformation of them with denser packing as a result. Similar phenomena probably cause disturbances also in other chromatographic processes.

In all separations reported in this paper the values of the distribution coefficient have been zero for the large molecular species and about 0.8 for the small ones. Thus the sample volume has to be smaller than $0.8 V_t$. In the experiment illustrated in Fig. 4B the sample volume was $0.75 V_t$ and an almost complete separation was still obtained. It should be observed, however, that the viscosity of the sample was very near to that of the eluant. With large sample volumes the dilution of the components becomes small. In the cited experiment the protein was diluted 1.25 times and the salt 1.35 times.

When the viscosity is low the concentration of the sample appears to be of little importance. Observations in favour of this view have been reported[8,12].

In the experiments reported the solutes separated differed very much in molecular size, and the results are comparable to those obtained with dialysis. In fact, the process may be considered as a multistage counter-current dialysis, in which the gel particles replace the dialysis bags. It was therefore of interest to see whether the effect on the excluded solute was similar to that obtained in dialysis. In all experiments with low-viscosity samples the salt present was completely removed. The conductivity and pH were also studied and it was found that the pH was adjusted to that of the buffer, provided that the stationary phase contained enough buffer ions to titrate the protein. The effects on the conductivity indicated that a Donnan equilibrium had been established. Experiments with polyelectrolytes and distilled water as eluant have shown that extraneous ions are removed, but the counter-ions remained with the polyelectrolyte. Thus all evidence hitherto collected indicates that the results are equivalent to an exhaustive dialysis. An important difference from the latter procedure is that a gel filtration experiment may be performed in a much shorter time.

A complete removal of salts from a protein is possible if water is the eluant. In many cases, for instance with blood serum a precipitate may develop which clogs the column. To prevent such complications a volatile buffer is advantageously used since it may be removed by lyophilization or evaporation.

SUMMARY

1. A study of the gel filtration method has been made in order to find the optimal conditions for removing salts from proteins.

2. The column efficiency increases with decreasing particle size and flow rate.

3. The viscosity of the sample rather than the concentration is a limiting factor.

4. Gel filtration of a protein solution is equivalent to an exhaustive dialysis but it can be carried out in a much shorter time.

5. The mechanism of the process is discussed.

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