A COLUMN DESIGN FOR REVERSE-FLOW SEPHADEX GEL PERMEATION CHROMATOGRAPHY*,**

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INTRODUCTION

The use of cross-linked dextran gels (Sephadex***) for molecular sieve fractionation has achieved wide popularity. The sieve properties of a particular gel are a function of the porosity of the gel particle, which in turn is determined by the degree of cross-linking between dextran chains. Of the commercially available materials, Sephadex G-200 has the largest pore size. This material allows the separation of spherical particles with molecular weights above 200,000 or asymmetric particles of equivalent Stokes radii[1]. As discussed by FLODIN[2], for a given gel particle size, separation is a direct function of the number of "theoretical plates". The number of "theoretical plates", N, is given by the formula, $N = (L/\mathrm{EHTP})$, where L is the column length and EHTP is the equivalent height of a theoretical plate, which, for a given particle size, can be decreased by reducing the flow rate.

Because of the great compressibility of the large pore-sized Sephadex G-200 particles, increasing the bed height can result in flow rates so slow as to be impractical. Moreover, many workers have observed the reduction of flow rate with repeated use of G-200 columns. In the course of studies on the separation of various proteins by G-200, we[3] began using the technique of reverse-flow (solvent flow up the column) in order to minimize or even avoid the compaction of the gel material. Independently, PORATH AND BENNICH[4], also dealing with the problem of gel compaction, described the use of reverse-flow in conjunction with the technique of recycling chromatography. At that time we adopted their column design, but subsequent experience led to major modifications resulting in the design reported in the present paper.

The reverse-flow technique has several distinct advantages. It enables one to use long columns of G-200 repeatedly while maintaining the same flow rate. Furthermore, since solvent flow is directed against the field of gravity, formation and maintenance of sharp sample bands can readily be achieved.

This paper presents a description of the reverse-flow column in use at present in this laboratory, the method of handling the G-200 in the formation of the column bed and the technique of sample application.

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** See MOORE[5] for a discussion of the term "gel permeation".

*** Obtained from Pharmacia Fine Chemical Co., New York 17, N.Y.

COLUMN DESIGN

Fig. 1 presents a diagram of the column. It consists of a lucite cylinder fitted at each end with a plunger, stem, and handle made of polyvinylchloride (PVC), which is much easier to machine than lucite. The face of the plunger has a funnel-shaped recess which directs the flow of liquid into the outlet channel. A disc of hydrophilic porous polyethylene* is inserted into a Teflon gasket and this assembly, in turn, is inserted into the recess in the face of the plunger. The Teflon gasket is employed in order to prevent leakage around the edges of the porous disc, which was

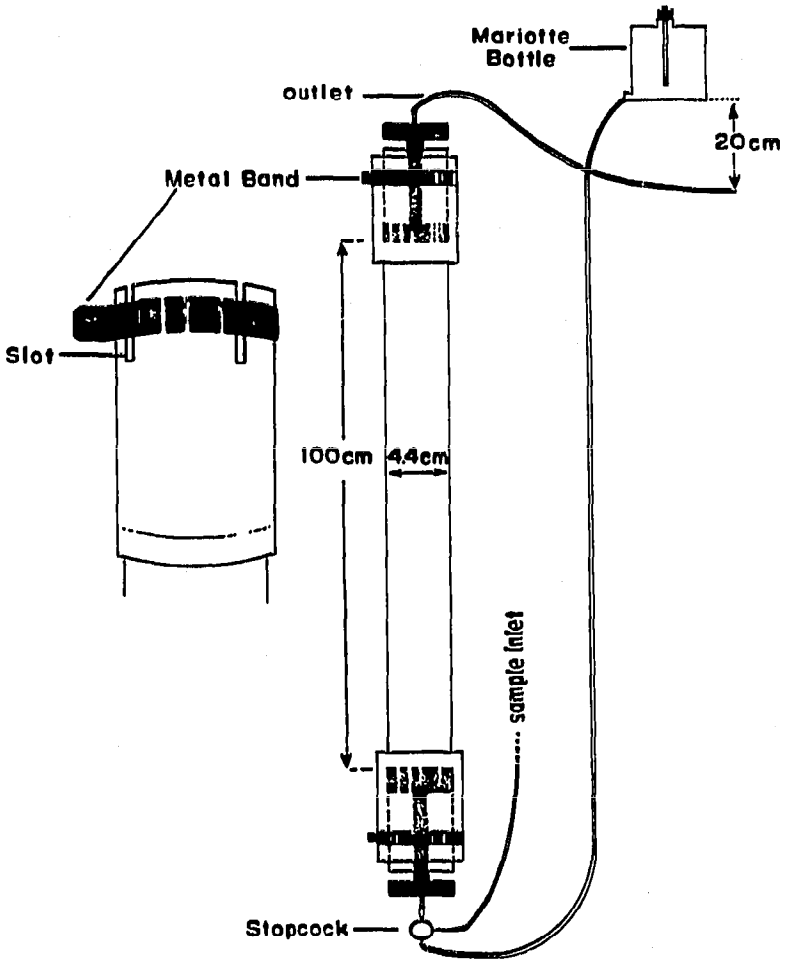

Fig. 1. Diagram of reverse-flow column. Left: Diagram of slotted column end with clamp.

observed when the porous polyethylene was directly fitted into the plunger. Hydrophilic porous polyethylene was chosen over other porous plastics because of its more rapid water flow rate. A lucite collar is threaded onto the stem of the plunger and acts as a guide upon insertion of the plunger into the column. The walls of the column are slotted at both ends. This permits the plunger assembly (Fig. 2) to be secured by compressing the column walls against the lucite collar by means of an adjustable metal band. The portions of the column into which the plunger assemblies are in-

* Obtained from Porex Materials Corp., Fairburn, Ga.

serted are double-walled in order to withstand the strains resulting from the tightening of the metal band. Prior to use, the porous filter is cleaned with a detergent and then thoroughly washed by passage of water through the plunger assembly channel. Boiled latex tubing, $^1/_{16}$-in. I.D. and $^1/_{16}$-in. wall thickness, is slipped over the tips. The use of latex tubing is preferable because the flow rate is faster with rubber, which is wettable, than with any of the plastics, which are not.

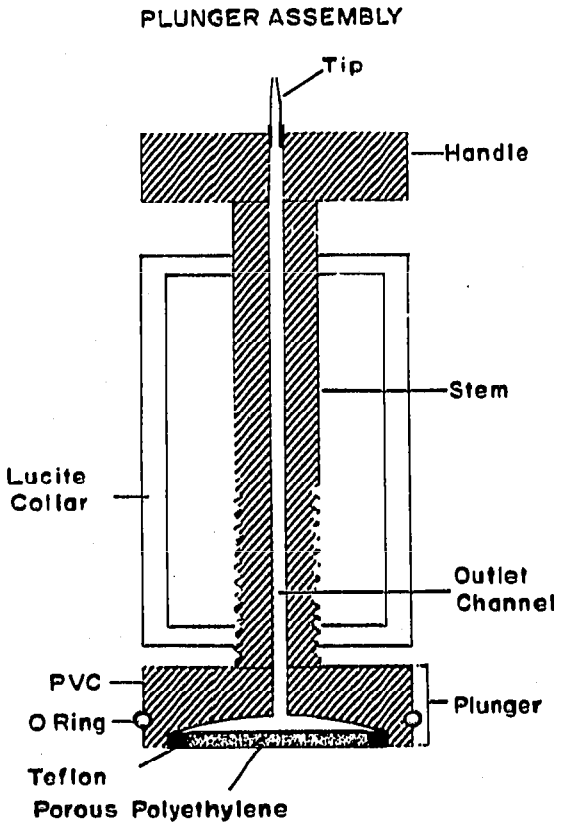

Fig. 2. Diagram of plunger assembly.

FORMATION OF THE COLUMN BED

Sephadex G-200 is passed through a set of U.S. Standard sieves. The 140–200 mesh (100–74 μ) fraction* is equilibrated at room temperature with gentle stirring for at least 72 h in the buffer to be used. Filling of the column is done at room temperature. A plunger assembly is inserted into the bottom of the column and the metal band is tightened. The filling vessel (Fig. 3) is put into the top of the column, which is then filled with buffer warmed to 40–50°. A small amount of coarse Sephadex G-25 (50 mesh, 300 μ) is added to form a $^1/_4$ cm layer on top of the filter, in order to prevent any of the finer G-200 particles from clogging the pores of the filter. After the G-25 has settled, about $^1/_3$ of the buffer in the column is allowed to flow out, so as to improve the packing of the layer of G-25. The outlet tubing is then closed and the column refilled with warmed buffer.

The suspension of G-200 is poured into the filling vessel and stirred with a

* For finer resolution the 200–270 mesh fraction should be used.

"serpentine" or paddle stirrer which reaches into the column. In agreement with BELING[6], it is essential for uniform packing of the bed material that the liquid in the entire column should be agitated by alternating the direction of rotation of the stirrer. This is accomplished by attaching the stirrer to a reversing motor programmed* to change the direction of rotation at 2-min intervals. Agitation of this type reduces the tendency of the gel particles to pile up along the column walls. The gel particles are allowed to settle until a 3–4 cm layer is formed. The outlet tubing is raised so that the distance between the buffer level in the filling vessel and the end of the

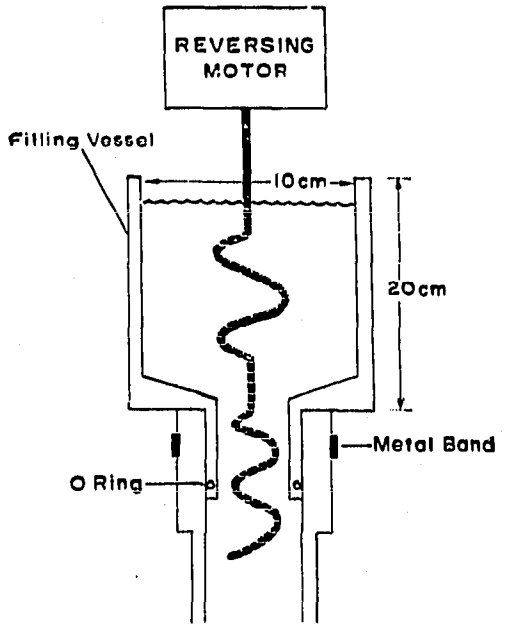

Fig. 3. Diagram of filling vessel with stirrers.

outlet tube is approximately 20 cm. The fluid is now allowed to flow at the rate of 35–40 ml/h under a 20 cm hydrostatic head. After the column is packed to the necessary level, it is moved into the cold room (+4°) where the column bed contracts slightly with temperature equilibration. Buffer is then run through the column for a period of 12–18 h, maintaining the same hydrostatic head. A $^1/_4$ cm layer of coarse G-25 (50 mesh, 300 μ) is then applied to the top of the bed of G-200. The flow is stopped and the column is allowed to equilibrate overnight. During this equilibration period the column bed expands. This last point is extremely important, for if the expansion is not allowed to occur, compaction of the bed will result with time. With a layer of buffer on top of the bed surface, the plunger assembly is carefully inserted to avoid trapping air bubbles and then pushed down to a level just above the bed surface. The plunger is then brought into contact with the bed surface by one or two turns of the handle on the threaded stem.

SAMPLE APPLICATION

The application of the sample from below has the distinct advantage of allowing the formation of a well-defined sample band. This condition is accomplished by es-

* Available from Technical Services, Cambridge 41, Mass.

tablishing density shelves at the upper and lower boundaries of the sample band, resulting in boundary stabilization. In general, the sample is denser than the elution buffer (if not, it can be made so by the addition of sucrose), so that the upper boundary of the sample zone is stabilized by the density shelf. In order to stabilize the lower boundary of the sample zone, the sample is followed by a denser solution made of buffer and sucrose or an inert material of high molecular weight, such as Ficoll or Dextran 10*. The polymer is used rather than sucrose in those cases where one wishes to avoid the "droplet effect" reported and discussed by SVENSSON *et al.*[7,8]. The choice of Dextran 10 or Ficoll is determined by the molecular size of the material under investigation. Ficoll (mol. wt. 400,000) is employed when materials which are not excluded from the gel particles are being studied. Dextran 10 (mol. wt. 10,000) is used when materials of greater molecular size are under investigation.

Application of the sample to the bottom of the column bed is facilitated by the use of a plastic three-way micro-stopcock. As shown in Fig. 1, the micro-stopcock is placed immediately below the bottom of the column in order to avoid mixing of the sample solution with the buffer of lower density. Care must be exercised to prevent the entrance of air into the tubing which leads into the column. Prior to application, the sample is filtered in order to remove any particulate matter which might clog the porous filter. After filtration, the sample is slowly fed into the column by means of gravity or by a motor-driven syringe. The stop-cock is closed and the "sample tubing" disconnected. New tubing is connected, and 10–15 ml of the high-density solution is slowly fed into the column. The stop-cock is then set so that the elution buffer enters the column from a Mariotte bottle so positioned that there is a hydrostatic head of approximately 20 cm across the column. The flow rate is regulated by means of a screw clamp on the exit tubing.

DISCUSSION

The modifications of the column design of PORATH AND BENNICH[4] described above facilitate assembly of the column. The presence of the lucite collar permits the guided insertion of the plunger assembly, eliminating the disturbance of the gel bed surface occasionally observed with the other column design. The method of securing the plunger assemblies (slots and clamps) eliminates the problems of machining and maintenance of lucite/lucite threads. The threading of the PVC stem through the lucite collar presents no problem. Furthermore, the accurate machining necessary for the leak-proof fitting of the filter disc is readily obtainable with PVC as opposed to lucite. The use of hydrophilic porous polyethylene contributes greatly to the reduction of the resistance to flow and in this regard is superior to other porous plastics.

Employing the column and techniques described in this report, a 4.4 cm × 100 cm column of Sephadex G-200 has been in continuous use for over 4 months with no change in flow rate (25 ml/h). The systems subjected to gel permeation chromatography have included human serum, crude collagenase preparations, vitreous and crude actin preparations. The column effluents have been monitored by continuous flow instruments which measure U.V. absorption, U.V. fluorescence and

* Obtained from Pharmacia Fine Chemical Co., New York 17, N.Y.

relative refractive index. When such equipment is placed in the flow line, the Mariotte bottle must be raised in order to compensate for the additional resistance of the tubing added to the system.

The separation patterns obtained are similar to those reported by other workers[2,9]. The patterns obtained with the continuous flow refractometer* are of interest and will be reported in a later paper. Fluorescence was measured by a Turner continuous flow fluorometer, Model 111, which was modified so as to automatically attenuate the instrument sensitivity. This modification** will be described in a later paper.

In a very recent article, BROMAN AND KJELLIN[10] described a plunger somewhat similar to ours for use in a reverse-flow column of Sephadex G-100.

The column described in this paper is commercially available from Future Plastics, Inc., 152 Columbia Street, Cambridge 39, Mass., U.S.A.

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SUMMARY

The design of a column for reverse-flow chromatography has been presented. The column has been employed for gel permeation chromatography with Sephadex G-200. Techniques for the formation of a column bed of G-200 are described as well as a method for applying the sample to the column. The techniques described allow for the continuous use of the G-200 column without changes in the flow rate with extended use.

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* Model R4, available from Waters Associates, Framingham, Mass.
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