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Gel chromatography: improved resolution through compressed beds

Since its introduction a decade ago, gel chromatography has found such widespread use in the purification and characterization of macromolecules that the technique has become known as the poor man's ultracentrifuge. Our experiments have shown that by simple longitudinal compression of a column of gel particles, the resolving power of a gel column can be at least doubled without causing a serious increase in the resistance to flow through the column. The shortened bed also reduces both the column size and the time required to achieve a desired degree of separation.

In its most common form, gel chromatography separates mixtures of molecules according to their size and shape by an elution technique using a column filled with small particles of gel [1,2]. A small portion of the mixture to be separated is applied to the top of the bed of gel particles; elution with solvent or buffer then causes different components of the mixture to migrate through the column at different rates. Solutes too large to penetrate the pores of the gel remain in the mobile solvent and are eluted from the column by a volume of solvent approximately equal to the void volume of the column (that portion of the column not occupied by gel particles). Smaller solutes diffuse into the stationary gel particles and their passage through the column is thus retarded. Because there is a distribution of pore sizes in the gel, the smallest solutes are retarded more than solutes of intermediate size. In the absence of adsorptive effects, the smallest solutes are eluted from the column by a volume of solvent slightly less than the total volume of the gel bed[1,2].

Because the great majority of evidence favors a steric exclusion mechanism for gel separations[1,2], we decided to see whether decreasing the void fraction in a gel bed would cause a proportionate reduction in the elution volume of totally excluded solutes and an increase in the resolving power for solutes of intermediate size. Although we had anticipated increased broadening of the solute zones due to the distortion of the gel particles by the bed compression, we were pleasantly surprised to discover that within limits, bed compression sharpened the zones instead.

Methods

The experiments were conducted in a jacketed glass column (Sephadex Laboratory Column Type K25/45, Pharmacia Fine Chemicals, Inc., 800 Centennial Ave., Piscataway, N.J. 08854). Water from a bath was circulated through the jacket to maintain the temperature constant and near 26°. The column is 2.5 cm in diameter and can accommodate a maximum bed length of 45 cm. An adjustable piston (Sephadex Flow Adaptor, designed for upward flow or recycling chromatography) was inserted into the upper end of the column and could be adjusted downward to remain flush against the top of the gel bed. Constant flow of eluting solvent to the column was achieved by gravity feed from a solvent reservoir. The eluant flow rate through the column was varied by raising or lowering the reservoir. A commercial dextran gel cross-linked with epichlorohydrin (Sephadex G-50, Coarse Grade, Lot 8146, Pharmacia Fine Chemicals, Inc.) was used in the experiments reported here. The dry particles ranged from about 0.1 to about 0.3 mm in diameter. On swelling in water, their diameter increased slightly more than two-fold. The gel particles were swollen in distilled

water for 4 h prior to packing the column with the gel. Distilled water was also used as the eluant. The column was packed by pouring a slurry of swollen gel particles into the column initially filled with water but draining at a constant and low flow rate[1,3]. After allowing the particles to settle, the flow rate was increased to 2.0 ml/min (this value was used in all elution experiments). The piston was then adjusted downward to give a flush fit with the top of the settled gel bed and elution experiments were begun. After duplicate runs at this bed height, which was slightly less than the freely-settled bed height, the flow rate to the column was increased several-fold to further compress the gel bed. Additional elution experiments were then conducted with the partially compressed bed and the procedure was then repeated using a still higher flow rate to achieve further bed compression.

TABLE I

ELUTION PARAMETERS IN COMPRESSED GEL BEDS

L (cm)	V_e (ml)		H (mm)		R_s	$\Delta P/L$
	Dextran	$Ni(NO_3)_2$	Dextran	$Ni(NO_3)_2$		(cm H_2O/cm)
35.8	73.6	153.3	2.12	4.13	7.2	0.087
	72.3		1.74		7.5	
33.2	58.3	165.4	1.82	1.94	12.5	0.23
	58.2	163.3	1.44	2.56	11.5	
31.0	47.4	149.6	1.43	2.03	13.2	0.39
	46.9	151.4	1.17	2.04	13.6	
28.8	37.9	140.3	1.52	1.62	15.1	0.47
	38.0	139.1	1.42	1.95	14.1	

In each elution experiment, samples of 2 ml of distilled water containing 2.48 % (w/v) Blue Dextran 2000 (BD) (Pharmacia, Lot 2012) and 0.5 M $Ni(NO_3)_2 \cdot 6H_2O$ (NN) (Analytical Reagent, Lot WHRS, Mallinckrodt Chemical Works, St. Louis) were separated with the column. Samples were introduced by using a capillary sampling valve mounted just above the inlet to the column. The column effluent was sampled using an electronic fraction collector (ISCO Model 270, Instrumentation Specialties Company, Inc., Lincoln, Nebr. 68507). Dead volumes were minimized throughout the apparatus; the residual end effects were measured independently and used to correct the elution data by standard methods[1,4-6]. Concentrations of Blue Dextran and nickelous nitrate were determined by spectrophotometry. Pressure drop across the column was measured as the difference between the height of the reservoir and the height of the column outlet, less the pressure difference due to end effects. Viscosities were measured with an Ostwald viscometer standardized with distilled water.

Results

The results of duplicate experiments at four different bed compressions are summarized in Table I. The elution volume (V_e) and the height equivalent to a theoretical plate (H) were calculated using eqns. (1) and (2), respectively.

$$V_{ej} = \sum_{\text{peak } j} C_{ij}V_i / \sum_{\text{peak } j} C_{ij} \quad (1)$$

where

C_{ij} = concentration of component j in the ith fraction
V_i = volume of ith fraction

$$H_j = L \left[\frac{V_{ej}}{\sqrt{\{(\sum_{\text{peak } j} C_{ij}(V_i - V_{ej})^2 / \sum_{\text{peak } j} C_{ij}) - \text{End effect correction}\}}} \right]^{-2} \quad (2)$$

where

L = height of gel bed

The end effect correction in eqn. (2) usually changed the value of H(BD) by about 5 % and H(NN) by less than 1 %. The resolution (R_s) is defined by eqn. (3).

$$R_s = \frac{2[V_e(\text{NN}) - V_e(\text{BD})]}{V_e(\text{NN})\ \sqrt{\{H(\text{NN})/L\}} + V_e(\text{BD})\ \sqrt{\{H(\text{BD})/L\}}} \quad (3)$$

All calculations were made with an IBM 360/65 computer.

The first row of values in Table I correspond to a bed compression of about 90 to 95 % of the freely-settled bed volume. Assuming that the Blue Dextran was totally excluded from the gel particles because of its high molecular weight (*ca.* 2,000,000), the void fraction in the gel bed may be estimated as 0.42. (The void fraction is calculated as the ratio of the V_e (BD) to the total volume of the gel bed.) At the greatest degree of bed compression reported here (L = 28.8 cm), the void fraction was approximately 0.27.

Examination of Table I shows that as the gel bed was compressed to about 75 % of the freely-settled volume, the elution volume of totally excluded Blue Dextran was nearly halved, while the elution volume of the (presumably) totally penetrating nickelous nitrate was reduced by a small amount (relative to its total elution volume). Comparison of the differences in the elution volumes of the two components suggests that a small decrease (about 5 %) in the inner volume of the gel may have occurred as a result of the compression, although further experiments are necessary to establish this definitely. Initial theoretical plate heights were comparable to those obtained by other investigations under similar conditions[1,3]; plate heights were decreased significantly by compression, and we feel that reduced channeling more than offset any shape effects that would have increased plate heights on compression. Combining these various effects, the resolution of the column was doubled by the compression, despite a 25 % decrease in the bed length. The only undesirable effect of the compression was a five-fold increase in the pressure drop through the bed, but even at its highest value, the total pressure drop was only 13.5 cm of water.

These results demonstrate the value of gel compression to gel chromatography with Sephadex G-50. If this technique were attempted with gels such as Sephadex G-200, agarose gels, or lightly cross-linked polyacrylamide gels, the much softer nature of these gels could be expected to cause very high pressure drops, necessitating the use of special equipment. In fact, a large share of the success of high-pressure gel chromatography with highly compressible gels probably reflects a resolution enhancement mechanism very similar to that found here with a simpler system. Extension of

the method to much harder gels may require use of a pump, such as the system described by HEITZ AND COUPEK[6], for compressive packing of the column. A subsequent publication will describe additional experiments at a wider range of gel compressions using a variety of gels, particle sizes, flow rates, and compression techniques[7].

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Miniature column chromatography of hemoglobins

Liquid chromatography, an extremely valuable tool for the study of biological compounds, is more easily adapted to the quantitative isolation of component compounds than is electrophoresis. However, chromatography usually requires more time, labor and material than does electrophoresis. To overcome these disadvantages, a simple chromatographic method has been developed using short, narrow bore columns that allow relatively rapid flow rates because of diminished back pressure. Although such a system can be used to study many substances, the separation of hemoglobins is reported here as an illustration of the efficacy of the technique.

Methods and material

Materials. The column, called a "minicolumn" (see Fig. 1), consists of a piece of glass tubing, approximately 2 mm I.D. and 150 mm long, with Technicon polyethylene nipples, size N-5, abutted snugly against each end by the use of vinyl tubing. A tiny plug of glass wool inserted in the column against the lower polyethylene nipple supports the resin in the column.

Both diethylaminoethyl-cellulose (DEAE-cellulose) and carboxymethyl-cellulose (CM-cellulose) have been used as resins for packing the columns although CM-cellulose does not give as consistent results as does DEAE-cellulose. The DEAE-cellulose was Selectacel No. 72, type 40, purchased from Schleicher and Schüll and the CM-cellulose was Whatman CM32 or Bio-Rad Cellex CM. Since adequate removal of fines from CM32 was difficult, excessive back pressure was a problem at times. For some purposes Cellex CM, lot No. 5170, was satisfactory.

The buffers used in this work were those of HUISMAN *et al.*[1,2]. Since the buffer