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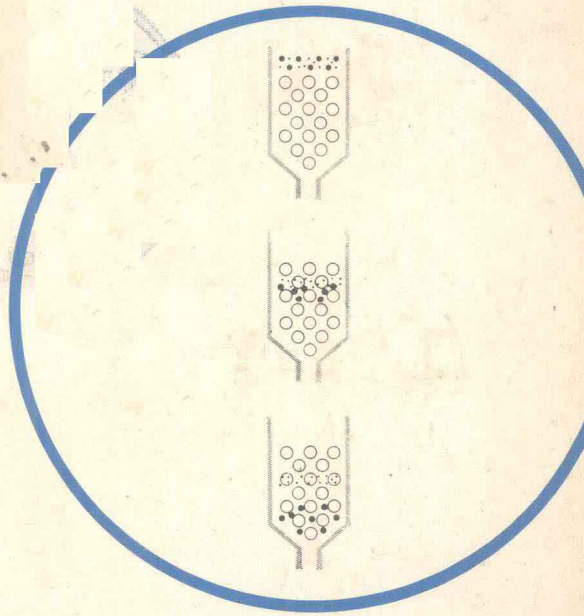
gel filtration chromatography

L. FISCHER



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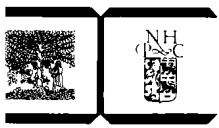
GEL FILTRATION CHROMATOGRAPHY

2nd Fully Revised Edition

L. Fischer

Sangtec Medical
Stockholm



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Part I Electrophoresis of proteins in polyacrylamide and starch gels
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by J. Clausen – 2nd Edition 1980

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Contractions used in the text:

DEAE	Diethylaminoethyl, charged group used in anion exchangers for biochemical purposes
DMSO	Dimethylsulphoxide
DMF	N, N-Dimethylformamide
DNA	Desoxyribonucleic acid
DNP	Dinitropheny – group used to stain proteins and amino acids
ECHO virus	commonly occurring human intestinal virus
NMP	N-Methylpyrollidone
RNA	Ribonucleic acid
Tris	Tris(hydroxymethyl)aminomethane

Contents

What is gel filtration chromatography?

1.1. Gel filtration chromatography – a biochemical separation method

The history of chemistry is to a remarkable extent the history of the separation and purification of substances from one another. In many discoveries the decisive step has been the right solution of a separation problem. Examples may be cited from every branch of chemistry, but the branch of chemistry that has relied most heavily on various separation methods is no doubt biochemistry. Although much interesting information can be obtained by observation of intact cells and organisms, it is also essential that these complex structures be taken apart and the various parts studied separately. However carefully the parts have been separated from the cells, a complex mixture of substances will always result. The success of further investigations will then depend on refined methods for separation.

The various separation methods available will sort the substances with respect to different parameters. In some cases the variable involved is easy to pinpoint. Isoelectric focusing (electrophoresis in stable *p*H gradients, see for instance Vesterberg and Svensson 1966; Haglund 1967) sorts the substances according to their isoelectric points while preparative ultracentrifugation fractionates them with respect to their diffusion coefficients, their masses and their buoyancy factors. In other cases it is more difficult to define the variables governing the separation. For precipitations it is very difficult to relate the order in which the molecules precipitate to the basic molecular parameters.

When separating a complex mixture it is essential that the different

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methods used should be based on different principles with as little overlap as possible. Thus, methods which ultimately depend on the same property of the substances, such as electrophoresis, ion exchange chromatography and isoelectric focusing, all take advantage of the charge on the molecules and it is unprofitable to apply all three in succession.

When gel filtration chromatography was introduced, it was found that the separation was based on a property that was new among biochemical fractionation methods: it is a separation method based on differences in molecular dimensions. It is true that the separation obtained with the preparative ultracentrifuge also depends on molecular mass, together with the diffusion coefficient and a buoyancy factor. This method is, however, rather complicated and does not give very sharp separations. It is limited to substances of high molecular mass. Ultracentrifugation also is very time-consuming and has a very limited capacity. Dialysis can also be said to give a separation according to molecular mass, although this separation is rather coarse.

Gel filtration chromatography therefore fills an inportant need and was rapidly accepted as one of the most important separation techniques in biochemistry. Many reviews have been published, presenting the method from different viewpoints. Most important are those by Porath and Flodin (1963), Determann (1967), Ackers (1970), Curling (1970), Reiland (1971), Freeman (1973), Cooper et al. (1973), Čoupek et al. (1975) and Determann and Brewer (1975).

1.2. The mechanism of gel filtration chromatography

As indicated by the name, gel filtration chromatography is a chromatographic technique. Before going into the more specialized subject of filtration chromatography, it seems appropriate to give a brief description of the mechanism by which separation is obtained in chromatography.

The tool for obtaining a separation is the chromatographic bed. The bed consists of minute particles usually packed into a tube. In the more specialized technique of thin-layer chromatography, the particles are

spread in a layer over a solid support. The space between the particles is occupied by a liquid which is made to flow through the bed. The substances to be separated are carried through the bed by the flow of the liquid. The stationary phase retards the progress of substances through the bed. Different substances are retarded to a varying extent and so migrate through the bed at different velocities. If two substances are applied at one end of the bed mixed with each other, they will leave the bed separated from one another, the faster moving leaving the bed first, followed by the slower moving substance.

The retardation is effected by partitioning of the substances between the liquid and the stationary phase. When a molecule is in the stationary phase, it does not move in the direction of the liquid flow, as all the flow of the liquid takes place between the gel grains and there is no bulk flow of liquid through the gel particles.

The rate at which a solute passes through the bed is proportional to the mean time, calculated on a statistical basis, that the molecules spend in the liquid between the gel grains. This average time is determined by the partition coefficient for the substances between the gel phase and the liquid phase, as the average for a given molecule over a period of time is equal to the average for a large number of molecules at a given time.

The characteristic property distinguishing gel filtration chromatography from other types of partition chromatography is that the grains that form the stationary phase in the chromatographic bed consist of an uncharged gel. This gel is swollen in the same solvent as that percolating through the bed. Typically, the gels consist of macromolecules with great affinity for the solvent used. These have been cross-linked to form a three-dimensional network, thereby making them insoluble. Instead of dissolving in the liquid, they swell, taking up large amounts of liquid.

Gel filtration chromatography separates substances according to their molecular size; large molecules will emerge first from the bed, while smaller molecules are retarded. Few substances will, however, be retarded so strongly that their elution volume will be higher than the total column volume. For many groups of substances a very close

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correlation is found between molecular mass (or molecular size) and elution behaviour, and for practical purposes the elution volume is usually determined entirely by the molecular mass. This can be explained by the simple model for gel filtration chromatography, introduced by Flodin (1962a). According to him, the partition coefficient of the solute between the gel phase and the liquid phase is governed exclusively by steric effects. He points out that the gel matrix occupies a great deal of space in the immediate environment of the cross-links. Large molecules cannot penetrate into these regions, while small molecules can approach the cross-links more closely. Small molecules have access to all the space between the chains of the gel matrix and will consequently be distributed fairly evenly between the free liquid and the liquid in the gel. Large molecules have access to less space within the gel, and the partition coefficient is shifted in favour of the liquid outside the gel grains. This induces large molecules to emerge from the chromatographic bed earlier than smaller ones.

Although the model of Flodin explains the elution behaviour of most substances, certain exceptions have been observed. The model does not account for elution volumes larger than the total volume of the liquid in the chromatographic bed (the sum of the amount of liquid in the gel and between the gel grains). Although few substrates are so strongly retarded that they are difficult to elute, elution volumes larger than this theoretical maximum have often been observed. Aromatic substances (including heterocyclic aromatic substances) are known to give elution volumes larger than those expected (Gelotte 1960). A number of other substances with higher elution volumes than those expected from the steric model have been reported: hydrophobic organic molecules, inorganic anions and cations are retarded by hydrophobic interaction effects in aqueous solutions; hydrogen bonds and hydrophilic character determine the interaction in organic solvents. Enzymes have been reported to be retarded due to the substrate similarity of the gel matrix.

If the separation were a steric effect only it would be described thermodynamically as an entropy effect as long as the swelling of the gel does not vary with the temperature. This would mean that tem-

perature effects would be absent. To a large extent this is the case for substances, that are eluted as expected from their molecular size and where interaction between the matrix and the solute does not seem to take place. In those cases, where solute-matrix interaction is observed, considerable temperature effects have also been observed.

Although deviations from the general pattern can thus be observed, the following picture of gel filtration chromatography will be sufficient for practical purposes: The molecules are carried down the chromatographic bed by the flow of the liquid between the gel grains. Large molecules are for steric reasons unable to penetrate into the gel grains and travel at the same rate as the liquid. The smaller molecules can penetrate into the gel grains. Molecules inside the gel grains are stationary. The average velocity in the direction of the flow is proportional to the time spent in the space between the gel grains. Small molecules will consequently travel at a lower rate. The principle is illustrated in fig. 1.1.

A strange deviation from the pattern that would be expected from this description of the mechanism is given by Wilson and Greenhouse

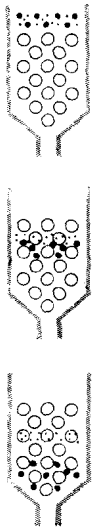

Fig. 1.1. Principle of gel filtration chromatography. A sample containing a mixture of small and large molecules is applied to a gel column. The small molecules (small dots) can pass into the gel beads (open circles) and lag behind the large molecules (large dots) that cannot penetrate into the beads. From 'Sephadex – gel filtration in theory and practice', by Pharmacia Fine Chemicals.

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(1976). They claim to have obtained a considerable separation of the radioactive iodine isotope ^{125}I and the natural isotope ^{127}I by simple-gel filtration chromatography on Sephadex G-10 or Sephadex G-15.

1.3. Characteristic properties of gel filtration chromatography

There are several reasons why gel filtration chromatography was very rapidly adopted as a standard procedure after its introduction around 1960. The following features are probably those which have contributed most strongly to its rapid progress:

Gel filtration chromatography is technically simple to perform. It is remarkably insensitive to composition of the eluant and to temperature. Even very labile compounds may be treated with little risk of destruction. The gel matrices in gel filtration chromatography generally do not cause denaturation, and the procedure can be performed under very mild conditions.

Gel filtration chromatography fractionates substances of very high molecular mass. By varying the contents of gel matrix in the gel, the fractionation range can be varied within wide limits. The most dense microreticular gels fractionate substances in the region below molecular mass 1 000; with other gels, fractionation ranges extending up to molecular masses of several millions can be attained.

With gel filtration chromatography certain standard problems in biochemistry can be solved in a very rational way. Well-known examples are desalting of solutions of protein and other high molecular mass substances, and determination of molecular masses of macromolecules, particularly proteins.

The chromatographic beds in gel filtration chromatography usually need no regeneration and can be used over and over again. The materials are remarkably stable, and the same gel can be used for years without change of chromatographic properties, provided microbial growth is avoided.

Basic concepts

2.1. *Some definitions*

Before going more deeply into the technical details of gel filtration chromatography, a short discussion of the terminology used may contribute to the understanding of the subject, especially for those who are not familiar with chromatography. Like many other branches of science, gel filtration chromatography has a nomenclature with a number of synonyms and ambiguities. A number of scientists have introduced nomenclatures of their own. In this work, the commonest terms will be used as far as possible. In certain cases common terms have been used rather restrictively to avoid ambiguity.

The technique itself has been given different names by scientists wanting to stress different aspects of the technique. Porath and Flodin (1959) called the technique *gel filtration*, while Fasold (1975) preferred to call it *molecular sieve filtration*. Steere and Ackers (1962) used the term *restricted diffusion chromatography*, Pedersen (1962) *exclusion chromatography*, Hjertén and Mosbach (1962) *molecular sieve chromatography*, Moore (1964) *gel permeation chromatography*, and Determann (1964) *gel chromatography*. The designation used in this book, *gel filtration chromatography*, is one of the terms suggested by the nomenclature standardizing committee in ASTM D-20.70, see Bly et al. (1971).

Two different principles have been used to designate chromatographic methods. The name can refer either to the material in the bed or to the mechanism by which separation is achieved. In gel filtration chromatography the mechanism is probably more complex than is

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implied by names relating the method entirely to steric mechanisms. Many cases are known where steric mechanisms alone cannot give the result obtained, and no entirely satisfactory account of gel filtration chromatographic mechanism has yet been reported which takes into account all the factors that should contribute to the separation. It is therefore rather arbitrary to make a methodological distinction between the cases where 'adsorption' and other possible mechanisms contribute to the separation, and those cases where only steric effects are involved. The two cases cannot be distinguished from the point of view of experimental technique and methodology. For this reason, the term gel chromatography would seem most adequate. As the use of this term has been specifically discouraged by the standardization committee, the term gel filtration chromatography has been adopted as one of the suggested terms that is most familiar and least misleading.

In the literature on gel filtration chromatography, the term *adsorption* has been used to describe a stronger retardation of a substance than that which would be expected from the steric model. The term may lead to some ambiguity. Adsorption chromatography is the term used where retardation of a substance on the chromatographic bed is caused by its adsorption on the surface of the particles, in contrast to partition chromatography, where the substances are distributed through the bulk of the stationary phase (Giddings 1965, p. 2 and 9). From this point of view, gel chromatography is a special case of partition chromatography (except possibly in some very few exceptional cases). Here, the term adsorption will be used very restrictively and only when its meaning is completely clear.

A certain confusion exists with regard to the use of the words *chromatographic bed, chromatographic tube* and *chromatographic column*. Here, the word chromatographic bed refers exclusively to the column packing material and the interstitial liquid. The word column will be used for the hardware encasing the chromatographic bed and also for the totality of hardware and bed where no confusion will arise. The word tube will be used exclusively as a designation for the hardware. The granulated or beaded gel material in the bed is designated *packing*

material or *bed material*. In theoretical discussions the word *stationary phase* is used, as the solutes are distributed through the whole gel phase, at least on a macroscopic scale. The liquid, percolating through the bed between the gel particles, is called *eluant* or, in theoretical discussions, *mobile phase*.

The solution of the substances to be separated is called *sample*. For the sake of variation, the substances to be separated will also be called *solutes*, although the term solute would, strictly speaking, refer to any substance dissolved in the liquid.

The SI system will be used. In this the molecular mass (not weight) is given without dimension or possibly in units (u). Pressures are given in pascal (Pa). 1 cm H_2O corresponds to about 100 Pa, 1 atm. is about 100 kPa, 1 psi is about 7 kPa.

2.2. *Variables used to characterize the bed*

When an experiment is described and the results are evaluated, it is necessary to select the relevant variables, as it would be impossible to describe a complete experimental set-up in every detail. In chromatographic experiments, the geometry of the bed and the flow rate are the most important variables determining the results.

The meaning of the terms *bed height* and *bed diameter* is quite clear and does not require much explanation. It should be pointed out, though, that the bed height (or the *bed length*) extends from the bed support to the upper gel surface (not to the top of the tube). In calculations of the experimental results, the volume of the chromatographic bed, V_t (the *total bed volume*), is one of the main variables. To calculate this from the length and diameter of the bed by the formula for the volume of a cylinder requires a very accurate determination of the diameter of the bed, as this parameter is squared and the relative error involved is doubled. It should also be remembered that the tube may be uneven, with a cross section that is not perfectly round, and that the cross-sectional area may vary over the length of the tube. For this reason it is recommended that in accurate work the volume of the tube should be determined separately (described in § 5.8.4).

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