LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

AN INTRODUCTION TO AFFINITY CHROMATOGRAPHY

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Preface

Within recent years, affinity chromatography has become established as one of the most potent separatory techniques available to the biochemist. The technique has been exploited in almost every area of biochemistry and its applications are rapidly infiltrating cellular biology, immunology, medicine and technology. Because of this explosive development, this book is not intended as a comprehensive account of all the available systems but rather as a guide to current trends. However, whilst the detailed methodology of these systems vary, they are based on common principles. The aim of this book is to delineate these common principles and show how they are applied in practice. Thus, following a brief introduction to chromatographic terminology, Chapter 2 deals with the basic concepts in the design of affinity adsorbents. Appreciation of these basic principles should permit the reader to select a suitable ligand for immobilisation for his particular system. Chapter 3 details most of the widely used immobilisation techniques and should thus carry the concept into the reality of a prospective adsorbent. The chromatographic techniques involved in testing the adsorbent and its subsequent application to the purification in question are given in Chapter 4. These three chapters contain all the information necessary to design, construct and run an affinity adsorbent. The remainder of the book deals with a variety of potential applications of the technique, both preparative and analytical, and a number of related techniques where the principle of affinity chromatography is exploited in slightly different ways. The examples have been chosen with a view to illustrating some of the parameters involved in affinity

separations and no attempt has been made to present a comprehensive review of each subject. Where applicable, references are given to more complete reviews of each specific area.

March 1978 C.R. Lowe

An introduction to chromatographic logic

1.1. The principles of chromatography

The history of biochemistry to a large extent parallels the history of the resolution and isolation of substances from cellular sources. However carefully the cellular organisation is disrupted, a complex mixture of biochemical substances will always result. In many cases a research problem is only brought to fruition by judicious choice of separation methods. The success of future investigations will thus depend on refined techniques for the separation of sensitive biochemical substances.

The optimal resolution of a complex mixture of biochemical substances will largely reflect a combination of techniques which will sort the substances according to different principles. Thus a number of techniques such as electrophoresis, isoelectric precipitation and ion exchange chromatography exploit the overall charge of the molecules. Other techniques such as preparative ultracentrifugation fractionate the molecules according to their size or diffusion coefficient. A successful purification regimen will incorporate a combination of these techniques such that the complex mixture is successively fractionated according to several different molecular principles. Almost without exception, a preparative isolation scheme will involve some form of chromatography.

Chromatography involves the separation of the components of a mixture using a medium, the *stationary phase*, through which a flow of liquid, the *mobile phase*, is passed to achieve a differential migration of the components. In practice the separation is effected

on a chromatographic bed or column. The bed comprises minute particles of the chromatographic medium usually packed into a tube. The space between the particles is occupied by a liquid which is made to flow through the bed by gravity, pressure or some other mechanical means. The substances to be resolved are carried through the chromatographic bed by the flow of the mobile phase. The stationary phase retards the percolation of substances through the column, with different substances being retarded to differing degrees and thus migrating through the bed at different velocities. The chromatographic bed will thus resolve a multicomponent mixture by differential partioning of the components between the liquid mobile phase and the stationary phase. This type of chromatography is generally referred to as *partition chromatography* since the separation is achieved by partitioning of the substances between solvent or liquid immobilised on a solid such as cellulose, Sephadex, agarose or polyacrylamide and the liquid mobile phase which flows round the solid particles. The separation thus depends on solubility differences between the stationary and mobile phases and specific interactions between the components to be separated on the chromatographic medium or solvent are minimal. In contrast, the chromatographic medium in *adsorption chromatography* is designed or selected to interact more or less specifically with some or all of the components of the mixture to be resolved, and the liquid mobile phase is chosen to increase or decrease these specific interactions.

In principle, partition chromatography is based on the ideal thermodynamic behaviour of all the components involved, whilst in adsorption chromatography the opposite is the case. In practice, however, this distinction is rarely obtained since most natural substances interact with each other to some extent. Consequently, irrespective of the selected chromatographic technique, both partition and adsorption processes can mutually assist or interfere and thus assume importance in effecting a particular separation. This feature of chromatography makes the selection of chromatographic materials for a particular separation of great importance.

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1.2. Partition chromatography

For successful separations by partition chromatography, the stationary phase should be inert and non-adsorbing and be of an adequate mesh size to achieve a good balance between flow rate and resolution. The chromatographic support, often silica gel, alumina, cellulose powder or kieselguhr, may be used for the separation of both apolar and polar substances. For example, if the support is coated with a hydrophobic liquid such as benzene and the mobile phase is a hydrophilic solvent such as methanol or formamide, the chromatographic bed may be used to resolve apolar materials. In contrast, if the bed is coated with a more hydrophilic solvent such as *n*-butanol and water is used as the mobile phase the chromatographic bed will resolve more polar materials.

In this type of liquid–liquid partition chromatography the separation depends on differences in solubility between the stationary and mobile phases. The *partition coefficient, K*, is the ratio at equilibrium of the amounts of a substance dissolved in two immiscible solvents which are in contact. Thus, if an ideal substance *A* is dissolved in two ideal immiscible solvents, 1 and 2, then at equilibrium the partition coefficient, *K*, is a constant:

$$K = \frac{(A)_1}{(A)_2} = \frac{(C_A)_1}{(C_A)_2}$$

Needless to say, for the partition coefficient to be constant over a range of solute concentrations, adsorption effects must be minimal. If adsorption is not involved and the distribution of solute between the two phases is ideal then symmetrical peaks of the substances to be separated will be eluted from the bottom of the chromatographic bed. However, since equilibrium conditions must be relatively rapidly attained between the two phases and this involves diffusion in liquids, the flow rate of the mobile phase through the packed bed is important. It must be sufficiently low to allow equilibrium to be attained and yet not too low as to permit diffusion to broaden the peaks of the eluted materials.

Partition chromatography is employed largely for the separation of small molecular weight substances. Its application in the resolution of sensitive biochemical substances such as proteins has been largely superceded by more refined chromatographic techniques based on molecular principles other than solubility.

1.3. Gel filtration

Gel filtration (Flodin, 1962) is a technique of partition chromatography in which the partitioning is based on the molecular size of the substances to be separated rather than their solubility. The technique employs a mobile liquid phase and a stationary phase comprising the same liquid entrapped within an uncharged gel lattice. The chromatographic gels used in gel filtration comprise macromolecules with a high affinity for the solvent. These gels usually have a covalent cross-linked structure which forms a three-dimensional insoluble network. The gels are allowed to swell in the same liquid that percolates through the bed and in so doing imbibe large amounts of the liquid.

Gel filtration separates substances according to their molecular size; large molecules emerge from the bed first followed by the smaller molecules. For most practical purposes it suffices to say that the elution volume is determined almost entirely by the molecular weight. Flodin (1962) proposed a simple model for gel filtration to account for these observations. The partition coefficient of the solute between the gel phase and the mobile phase was deemed to be governed exclusively by steric effects. Large molecules cannot penetrate into regions close to the cross-links in the gel lattice because of steric obstruction. In contrast, small molecules can approach these regions more closely and thus have access to most of the space between the chains of the gel matrix. As a result small molecules are distributed fairly evenly between the free solvent and the solvent entrapped within the gel matrix, whilst large molecules are more restricted within the gel. The partition coefficient of large molecules is thus shifted in favour of the liquid outside the gel

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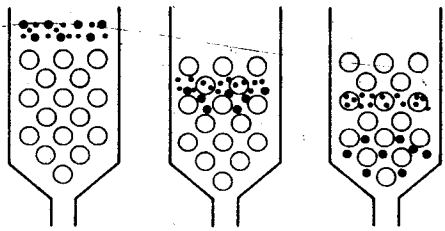

Fig. 1.1. The principle of gel filtration. A sample containing a mixture of large and small molecules is applied to the top of a gel column. The large dots represent large molecules, the small dots small molecules and the open circles the gel beads. As the elution proceeds, the small molecules penetrate the gel beads and lag behind the large molecules that pass round the beads. Reproduced with permission from *Sephadex®-Gel Filtration in Theory and Practice* by Pharmacia Fine Chemicals.

particles with the consequence that large molecules emerge from the gel bed earlier than small ones. Figure 1.1 illustrates the principle of gel filtration.

The solvent in a chromatographic column packed with swollen gel beads may be regarded as being in two phases (Flodin, 1961); in the spaces between the gel beads, the *void volume*, V_0, and entrapped within the gel matrix, the *internal volume*, V_i. A solute introduced into the column will equilibrate between the solvent contained in these two phases, although only a fraction of the internal volume, represented by the partition coefficient, K, is available to the solute. The total volume accessible to solute within the gel matrix is thus $K \times V_i$ and the solute will thus emerge in the mobile phase after a volume V_e, the *elution volume*, has been displaced

$$V_e = V_0 + K V_i$$

The partition coefficient,

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

is characteristic for chromatography of a given solute on a given gel under specified operating conditions and is independent of bed geometry. For solutes completely excluded from the internal volume

$K = 0$ and hence $V_e = V_0$, i.e., the solute emerges in the minimum possible volume, corresponding to the void volume. For very small molecules with unlimited access to the internal volume of the gel beads, K approaches 1 and V_e approaches a maximum value equivalent to $V_0 + V_i$.

In practice the volume of the solvent entrapped within the gel particles (V_i) is difficult to evaluate realistically. Consequently, an alternative means of expressing solute behaviour in terms of an *available partition coefficient*, K_{av}, is evoked. The internal volume, V_i, is replaced by the total volume of the gel ($V_i + V_g$), where V_g is the volume occupied by the gel matrix itself. Thus,

$$K_{av} = \left(\frac{V_e - V_0}{V_i + V_g}\right) = \left(\frac{V_e - V_0}{V_t - V_g}\right)$$

since the total packed volume of the gel bed, V_t, is given by:

$$V_t = V_0 + V_i + V_g$$

The available partition coefficient, K_{av}, is readily evaluated in terms of the bed parameters V_t and V_0, and the solute elution volume, V_e (Laurent and Killander, 1964). The total bed volume, V_t, may be deduced by calculation from the bed diameter and the bed height or by direct calibration with water. The column is filled with water and subsequently withdrawn from the column in portions and weighed. A plot of the weight of water against the level of water in the column will allow interpolation of the volume of any given bed height. The void volume, V_0, may be determined by chromatography of a substance that is completely excluded from the gel beads and measurement of its elution volume. In practice, a polysaccharide with a weight average molecular weight of 2×10^6, Blue Dextran 2000, is commonly employed for this purpose and is commercially available. The most important variable to be measured is the elution volume of the solute of interest, V_e, which should, within limits, be independent of the flow rate through the bed. If, as under conditions of ideal chromatography, the elution profile is symmetrical, the elution volume, V_e, is the volume of liquid

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that has passed through the column between the application of the sample and the elution of the maximum concentration of the substance.

The chromatographic behaviour of a solute in gel filtration can be related to its molecular size or a molecular parameter closely associated with molecular size such as molecular weight or Stoke's radius. It can be empirically shown that a plot of elution volume, V_e, or a suitable function of it such as K_{av}, of a number of globular proteins was approximately a linear function of the logarithm of their molecular weights (Ackers, 1964; Andrews, 1964). This relationship is depicted in Fig. 1.2. Below a certain molecular weight the curve is almost horizontal and in this region all solutes are eluted close together in a volume which is maximal for the given bed geometry and approximately $V_0 + V_i$. The central part of the curve is inclined downwards in such a way that a variation in molecular weight corresponds to a significant alteration in the elution volume. This part of the curve represents the working or fractionation range of the gel. Clearly, a gel that has a steep curve within the working range will efficiently fractionate molecules within that range. For practical purposes, however, an acceptable compromise between steepness and fractionation range is required. At molecular weights above the fractionation range, the curve becomes horizontal again with all solutes in this region moving with the void volume. The molecules are so large that $K_{av} = 0$ and $V_e = V_0$. This point is termed the *exclusion limit* of the gel.

Gel filtration may be used to determine molecular weights of globular proteins by interpolating the elution volume or K_{av} of the unknown protein on a plot of K_{av} versus log molecular weight constructed with standard proteins of known molecular size. Siegel and Monty (1966) showed, however, that the elution volume of proteins are better correlated with Stoke's radii than with molecular weights. Consequently, the method assumes that the asymmetry and extent of hydration of the proteins being analysed and of the standard calibration proteins are approximately the same. This assumption appears to hold true for most globular proteins but not for proteins

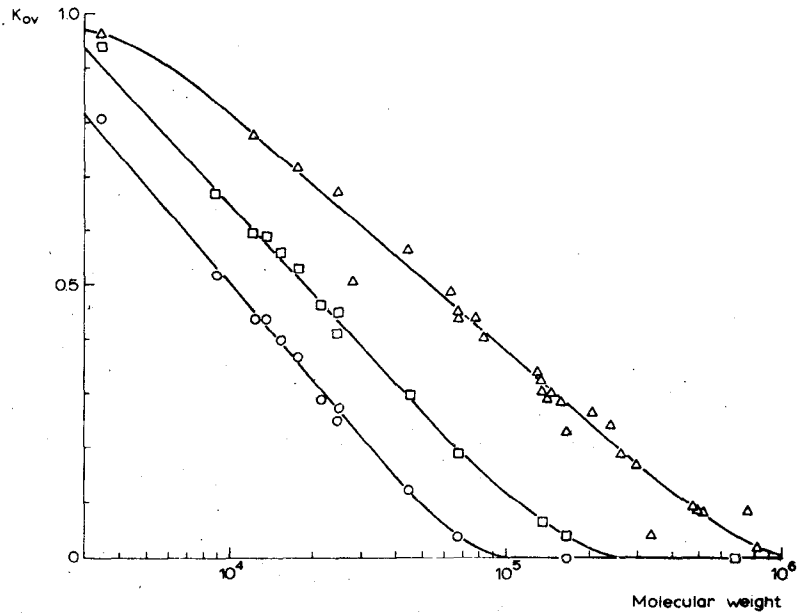

Fig. 1.2. The relationship between elution behaviour and molecular properties: K_{av} versus molecular weight for globular proteins on different types of Sephadex®. △ Sephadex G-200, □ Sephadex G-100, ○ Sephadex G-75. Reproduced with permission from *An Introduction to Gel Chromatography* by L. Fischer, North-Holland Publishing Co., Amsterdam, 1969.

containing large amounts of carbohydrate or for proteins that can interact with the gel matrix itself. Thus certain dextranases (Porath, 1968), polyglucanases (Pettersson, 1968) and plant haemagglutinins (So and Goldstein, 1968) may interact specifically and in some cases very strongly, with gel filtration matrices comprising polysaccharide backbones. In these cases the available partition coefficient, K_{av}, may exceed unity and become infinite in extreme cases. In such extreme cases the chromatography ceases to be partition chromatography and becomes adsorption chromatography. Full details of

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